ANXIETY DISORDERS

Edited by **Vladimir V. Kalinin**

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Edited by Vladimir V. Kalinin

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Preface

Anxiety, without a doubt, seems to belong to the most universal and ancient psychiatric phenomenon which has protective functions not only in *Homo sapiens,* but in other species of mammals too.

Sir Aubrey Lewis, one of the outstanding psychiatrists of twentieth century in his excellent work "Problems presented by the ambiguous word "anxiety" as used in psychopathology" (1967) has stressed the dual meaning of the term "anxiety" based on the etymology of this word. According to A. Lewis, the term "anxiety" implies not only the psychological phenomenon which signifies a state of apprehension, uncertainty, and fear resulting from the anticipation of a realistic or fantasized threatening event or situation, but also a combination of somatic and vegetative symptoms. The somatic or physical symptoms of anxiety are multifarious and include increased heart palpitation, headaches, dizziness or lightheadedness, nausea and/or vomiting, diarrhea, tingling, pale complexion, sweating, numbness, difficulty in breathing, and sensations of tightness in the chest, neck, shoulders, or hands. These symptoms are produced by the hormonal, muscular, and cardiovascular reactions involved in the fight-or-flight reaction.

According to another point of view, anxiety is considered as "a future-oriented mood state in which one is ready or prepared to attempt to cope with upcoming negative events", suggesting that it is a distinction between future vs. present dangers that divides anxiety and fear.

Anxiety is thought to be a normal reaction to stress. It may help a person to deal with a difficult situation, for example at work or at school, by prompting one to cope with it. But when anxiety becomes excessive, it may fall under the classification of an anxiety disorder and in such a case a concern of psychiatrists is required.

During the last 2-3 decades drastic research progress in anxiety issues has been achieved. It concerns mostly the study of different subtypes of anxiety and their treatment. Nevertheless, the data on anxiety pathogenesis is less elaborated, although here a multidimensional approach exists. It includes neurochemistry, pathophysiology, endocrinology and psychopharmacology. Again, we are able to recognize the multifarious sense of anxiety, and the present collective monograph

composed of 16 separate chapters depicts the different aspects of anxiety. A great part of book includes chapters on neurochemistry, physiology and pharmacology of anxiety. The novel data on psychopathology and clinical signs of anxiety and its relationship with other psychopathological phenomena is also presented.

The current monograph may represent an interest and be of practical use not only for clinicians but for a broad range of specialists, including biochemists, physiologists, pharmacologists and specialists in veterinary.

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1

A Probable Etiology and Pathomechanism of Arousal and Anxiety on Cellular Level - Is It the Key for Recovering from Exaggerated Anxiety?

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1. Introduction

How can stress of life evoke anxiety through the rather simple second messenger system? What is the main difference between acute stress response of humans and wild animals? What are the role of catecholamines and the alterations of carbon dioxide levels in anxiety disorder? How is it possible, that the same kind of stress causes very different symptoms and disorders in different people? How does the Path of Least Resistance work, and what is its importance? Do diseases of any etiology have a common pathogenetic root as a manifestation of the Second Law of Thermodynamics? What is the difference between functional and organic brain disorders at the cellular level? What is the pathomechanism of arousal and anxiety on cellular level? How does personality and behaviour affect one`s exposure to diseases? These are not easy questions, and we don't expect to find perfectly accurate answers to all of them. Especially not as we consider, that several decades were not enough to resolve the debate whether hypocapnia would cause anxiety, anxiety would evoke hypocapnia, or they would be mutually correlated making both statements true (Meuret et al., 2005). Although there is a huge amount of controversial data available that cause uncertainty, the authors try to answer these questions.

The BASIC idea of Sick Cell Syndrome is connected to Elkinton (1956). A modified universal disease model of Sick Cell Syndrome was constructed by author Sikter (published in 2007, Hungarian). The model is based on the Second Law of Thermodynamics. It is theoretical and practical as well. Adapting it properly may be a key for treating anxiety disorder up to "restitutio ad integrum". Since Kerr et al`s 1937 publication (as cited in Lum, 1981) there has been a long lasting dispute those who believe anxiety is caused by the lack of carbon dioxide, and those who insist that anxiety induces hypocapnia. Lum wrote the following in an editorial: "Although Kerr et al. (1937) had pointed out that the clinical manifestations of anxiety state were produced by hyperventilation, it was Rice (1950) who turned this concept upside down by stating that the anxiety was produced by the symptoms and, furthermore, that patients could be cured by eliminating faulty breathing habits. Lewis (1964) identified the role of anxiety as a trigger, rather than the prime cause. Given habitual hyperventilation, a variety of triggers, psychic or somatic, can initiate the vicious cycle of increased breathing,

symptoms, anxiety arising from symptoms exacerbating hyperventilation and thus generating more symptoms and more anxiety." Shortly after the conceptualization of panic disorder in the DSM III (1980), it became evident that there is an overlap between symptoms of the hyperventilation syndrome and panic disorder (Cowly & Roy-Byrne, 1987). Though psychiatrists see essential differences between the hyperventilation syndrome and anxiety disorder, practitioners of related fields do not (Han et al., 1997). "The patients in the present study (hyperventilators and patients with anxiety disorders) were diagnosed on different bases. However, the breathing pattern of patients with anxiety and hyperventilation syndrome is similar." The authors of this chapter argue that the truth is somewhere in the middle; that none of the original statements are false, and that the nature of the syndrome is best described by the vicious cycle concept of Lewis (1964 as cited in Lum, 1981). Hypocapnia can elicit somatic sensations, and the fear from these bodily sensations is capable to generate anxiety. On the other hand, anxiety is able to provoke hypocapnia, thus providing conditions for a vicious cycle.

2. Supposed mechanism of arousal. Is arousal linked to intracellular pH?

The roles of Ca^{2+} current (as second messenger) in the release of neurotransmitters and in the action potential of neurons (Neher, 1998) are well known, but less is the fact, that the low cytosolic H^+ concentration (cytosolic alkalosis) influences Ca^{2+} conductance as second/third messenger (Tombaugh & Somjen, 1997). Intracellular pH is strictly regulated (Boron, 2004) in the brain cells as well, and even marginal change of H^+ concentration may cause big functional alterations in neurons. Carbon dioxide concentration is one of the most important factors that influence the intra- and extracellular pH. CO_2 is extremely diffusible and when the breathing pattern changes--i.e. breathing slows down or accelerates--it will results in rapid changes of H^+ concentration both the intracellular and extracellular compartment, practically in all tissues and practically at the same time. CO_2 passes through the cell-membranes very quickly, and it forms carbonic acid with H_2O molecules which results in more H^+ ions in the cytosol. On the other hand, ions, including the H^+ ion, move slowly through membranes; this is explained by the fact that when electrically charged ions become hydrated, their radius gets multiplied. Conversely, CO_2 doesn't have either of these attributes and it is also soluble in lipids. Breathing deeply or frequently, the pulse speeds up, suggesting that CO_2 has left the pacemaker cells of the heart, and the alkalic cytoplasm allows Ca^{2+} to enter into the cytosol. Breathing this way for a longer time, the pulse will slowly come back to the initial rate, because the organism compensates the alteration of pH in the cytosol. The paucity of H^+ in the cytosol increases conductance of Ca^{2+} and some other ions (Harvey et al., 1988), thus it increases contraction, metabolism and O_2 requirement of the tissues (Laffey et al., 2002), and also increases excitability both in peripheral and central neurons (Stenkamp et al., 2001). All these events can be explained by the simple fact, that the alkalosis increases transmembrane conductance of ions and consequently increases active ion-pumping mechanisms as well, (thus restoring the original ion status.). By contrast, acidosis decreases Ca^{2+} conductance (Tombaugh & Somjen, 1997), and it also decreases excitability of the neurons, and the decreased Ca^{2+} conductance can dramatically affect the neurotransmitter release (Dodge & Rahamimoff, 1967). In some cells the Ca^{2+} entry into the cytosol itself increases cytosolic H+ concentration, which physiological acidosis then limits the further Ca^{2+} entry. It is supposed to be a novel feedback mechanism (Tombaugh, 1998). Carbon dioxide is an important link between psyche and corpus. Our emotions, actual

spiritual status and personality influence our breathing patterns (i.e. breathing rarely, frequently, irregularly) causing pH alteration in the organism. In this manner, the actual cytosolic pH of neurons affects arousal by modifying Ca^{2+} conductance, establishing a particular feedback mechanism. The concentration of carbon dioxide can alter the whole organism at the same time because of its extremely high penetrance. If the the concentration thus altered endures for a long time (several hours to a week), the organism starts to "compensate". Stability of extra- and intracellular pH is of high priority. Renal function and various tissue buffer mechanisms (mostly) restore the pH in the intra- and extracellular space, but the concentration of other ions in the cytosol remains altered for longer. The development of the new ionmilieu needs 5-7 days (Gennari et al., 1972). The new ionmilieu of the cells differs from the physiological one (restoration of the original ionmilieu would take another 5-7 days at least). Then chronic hypocapnia or hypercapnia is followed by cascades that alter the whole ionmileu in the cells. They may even alter the neurotransmitter/endocrine status (Dodge et al., 1967; Bailey et al., 2003).

Human is a species especially vulnerable to the long-term alteration of carbon dioxide levels. The explanation of this is the fact that humans become hypo- or hypercapnic not only by organic diseases, but by mental disorders as well, and – most importantly -- because of learned behaviours. This latter can be dangerous, because it may result in diseases of civilization (Sikter et al., 2009). It is frequently asserted that it is the "stress of life" itself that causes diseases of civilization (induced by "stress-hormones") (Selye, 1956). This statement might be incorrect in that wild animals don't get diseases of civilization, (except if they are living near civilization) even though they are at least as much stressed as human beings are. Wild animals behave and react according to their instincts. According to our viewpoint, in this acute stress response the most important factors to consider are the strong catecholamine (e.g. noradrenaline) rush and acute hypocapnia. During this hyperarousal condition wild animals will fight or flee. This exertion results in increased carbon dioxide production and this in turn restores the biochemistry and physiology. Human hyperarousal stress response is biphasic, but it is also accompanied by hypocapnia. (See below.) In their response to stress human beings differ from wild animals in that their response to stress is more complex. They can mostly restrain their temper, thus often physical activity will not follow hyperarousal stress. Moreover, the enduring hypocapnia would result in ion-movements through membranes, causing metabolic, endocrine alterations, and illnesses because of the alteration of „milieu interieur". Namely, diseases of civilization are caused by the distress evoked by the lack of instinctive reaction to stress. Animals become anxious and depressed after chronic immobilization (Joo et al., 2009), while humans are usually immobilized by their "civilized behaviour", and social norms.

Cannon's (1929) work on "fight or flight" stress response continues to be valid. (Stress evokes catecholamine rush and hypocapnia at the same time, which together result in a very strong sympathetic answer, hyperarousal.) There are several differences in the case of primates (Bracha, 2004). Homo sapiens begins with an „initial freeze response". According to Bracha, it is because mammalian carnivores are capable to see moving objects only. This is a hypoventilation/hypoarousal state with decelerated pulse. The fear-stressed human „stops, looks, and listens." This initial answer can be followed by two alternative ways. It can be followed by a hypocapnic response (this way of the reaction becomes very similar to the known Cannonian "fight or flight" stress response). Second possibility is the „tonic immobility" (Bracha, 2004), or „defensive immobility" (Van Diest et al., 2009). This is a

confirmation of the initial freeze response to a deeper hypoarousal state. (It was reffered to as „freezing behaviour and playing dead" in the early literature.) This is an explicit hypocapnic state with decelerated pulse and immobility. The animal/human is vigilant but is not capable (has no energy) to „fight or flight", although it can switch to hyperventilate and become hyperaroused if it seems to be practical. The hypoarousal fear responses and the relating hypercapnic breathing patterns are poorly studied (Van Diest et al., 2009).
It is not a widely known fact that there is a feedback mechanism between the catecholamine levels of tissues and intracellular pH (Tenney, 1960). In acidic conditions responsiveness to catecholamines dramatically decreases while catecholamine output increases. In spite of these compensatory processes the sympathicotonia and arousal decrease (Kuijpers et al., 1989). By contrast, responsiveness to catecholemines, sympathicotonia and arousal increase in alkalosis, (even though catecholamine output slightly decreases). Catecholamines increase Na^+/H^+ exchange in the cells that causes alkalosis in cytosol, similarly to the effect of hypocapnia. It might not be a coincidence. The Cannonian "fight or flight" response is a strong hyperarousal reaction because both catecholaminemia and hypocapnia cause alkalosis in the cytosol. In the case of humans the initial hypercapnia generates heavy catecholamine output. Then the cells become alkalotic via hypocapnia and hypersensitive to catecholamines. This biphasic hyperarousal stress response is characteristic not only of primates but of several other animals (e.g. opossum, some fish species, amphibians, reptiles, birds). Also well established is the fact that in the case of the ptarmigan hen the two phases of stress response were very pronounced and well distinguishable (Steen et al., 1988). In the first phase of stress, the hen's respiratory and pulse rate would decelerate. After several minutes the hen would suddenly start to hyperventilate, and the hypoarousal condition would convert to a very vigorous hyperarousal with up to two to three times faster pulse. In this, the hypercapnic period of breathing was followed by a hypocapnic one, similarly to symptoms of human panic disorder. (See below.)

3. Anxiety. The importance of breathing irregularity

Both hyperarousal and anxiety may elicit a physiological "fight or flight" response, but there are essential differences between them. Arousal is physiological, while anxiety is rather a psychological phenomenon. Anxiety develops via psychogenic pathomechanism, while the high arousal is one of the most important triggers of anxiety. Anxiety can occur without exposure to an external stressor and it is usually longer lived than arousal. High arousal is a precondition of anxiety, although anxiety is also a result of learning/conditioning process. High arousal can lead to anxiety while low arousal level can result in depression. Modern neuroimaging techniques have made anxiety-research an objective science (Sehlmeyer et al., 2009). The authors of this chapter emphasize the importance of altering carbon dioxide level both in hyperarousal and anxiety. According to author Sikter, the irregular breathing patterns in the pathomechanism of anxiety are as important as hypocapnia itself. The breathing irregularity is especially high in panic disorder but is also present in GAD patient (Wilhelm, 2001b). "The control of breathing is a complex interplay that relies on many factors, including the bulbopontine respiratory network, central and peripheral chemoreceptor control, modulation of respiratory muscles by mechanoreceptors, and numerous suprapontine networks located in the limbic, cerebellar, and cortical areas" (Van Diest et al., 2009). Perhaps, the existence of too many breathing centers to guide respiration

is the most important reason for irregularity. Inspiratory reflex could come from any one of the breathing centers; a negative thought can evoke a deep sigh, etc. (Bruce & Daubenspeck, 1995 as cited in Roth, 2005). The faulty breathing pattern can become habitual (Mitchell 2003). Resulting increased CO_2 levels evoke catecholamine production, and then an abrupt decrease of pCO_2 causes hyperarousal. The quick alteration of carbon dioxide level could be followed by compensatory electrolyte transport and buffering mechanisms, but only with significant time lag which may be as long as a week. On the other hand frequently elevated carbon dioxide levels increase catecholamine output, which are not neutralized by compensatory decrease of pCO2 levels, so they enhance each other's effect. (See above). Other hormones (e.g. cortisol) also can play a role (Bailey et al., 2003; Gorman, 2003). The interaction between cortisol and noradrenaline is controversial – cortisol seems to attenuate noradrenaline induced panic symptoms (Vasa et al., 2009). Progesterone influences arousal level probably via altering carbon dioxide level, it is thought to be responsible mainly for the high female/male ratio of anxiety disorder (Saaresranta & Polo, 2002). All dynamic cells within the human organism (neurons, smooth-, skeletal- and cardiac muscle cells, pacemaker cells of heart) become activated parallel with arousal. This can result in somatic sensations and fear. Both reasonable and unreasonable fear can create vicious circles throughout the brain circuit. Misinterpretation of bodily sensations caused by hypocapnia and irregular breathing maintain and accelerate these vicious cycles; Pavlovian reflexes may develop. The alternating carbon dioxide, catecholamine, and emotional tension levels can interfere with each other, in this way the arousal can be sustained, which can maintain psychological uncertainty and provoke a vicious circle.

4. Is there a connection between tendency to hypocapnia and neuroticism?

Hypocapnia is typically associated with anxiety states (Grossman, 1983). On the other hand there is a great overlap between the hyperventilation syndrome and anxiety disorder, and especially panic disorder (Cowly & Roy-Byrne, 1987; Gardner, 1996; Han et al., 1997). There is also growing amount of evidence that there is an inverse correlation between pCO_2 and both of personality trait neuroticism and psychiatric morbidity (Stegen et al., 1998; 1999; 2000; 2001 as cited in Van Diest et al., 2003). A negative relationship of $FetCO_2$ (resting end-tidal fractional concentration of CO_2) was found with trait negative affectivity. "The existence and direction of an association between $FetCO_2$ and trait negative affectivity is clinically important for a number of topics in the field psychosomatic medicine."; "…an association of trait negative affectivity with $FetCO_2$ may help to clarify the hypothesized role of hyperventilation in quite a number of pathological conditions, such as panic disorder, somatization, chronic fatigue syndrome, chronic muscle pain, and multiple chemical sensitivity." (Van Diest et al., 2003). Shu et al., (2007) proved that neuroticism is linked with self-reported symptoms of anxiety in hyperventilation syndrome. A robust significance was found probable due to the clinically homogeneous sample, (similar aged males were observed during military training). On the other hand, Dhokalia et al. (1998) found positive correlation between neuroticism and $FetCO_2$ in nonclinical sample. Yet, other studies found no association between $FetCO_2$ and anxiety (Wientjes & Grossman, 1994 as cited in Van Diest et al., 2003). The effect of emotions on pCO_2 and breathing patterns is also known (Masaoka & Homma, 1999). One's sensitivity to carbon dioxide challenge may prove to be a useful test for psychosomatic investigations (Shershow et al., 1973). It was found that childhood anxiety disorders, particularly separation anxiety, are also associated with CO_2

hypersensitivity (Pine et al., 2000). Carbon dioxide hypersensitivity was not found specific to panic disorder, rather to anxiety disorder. It is a question whether breathing abnormalities (written in Wilhelm, 2001a, 2001b, 2001c; Meuret, 2005; Roth, 2005) induce personality trait neuroticism (through hypocapnia) or is it in reverse? Are breathing abnormalities reversible or not (Meuret et al., 2005)? Is neuroticism a reversible personality trait or not? According to a population-based twin study a substantial overlap was found between the genetic factors „that influence individual variation in neuroticism and those that increase liability across the internalizing disorders, helping to explain the high rates of comorbidity among the latter." (Hettema et al, 2006). The identical twins breathe in similar ways (Shea et al., 1989 as cited in Homma & Masaoka, 2008.) The negative affectivity, neuroticism could alter breathing pattern, because the breathing has plasticity (Mitchell & Johnson, 2003). It means that genes have important role in „neuroticism" but these genes are not specific for a special anxiety disorder. Possibly, is there only one (general) neurotic syndrome (Andrews et al., 1990)?

We have to agree with Van Diest et al. (2003) that while „hyperventilation is a well known response to stress and anxiety, less attention has been paid to the opposite response", i.e. to the end-tidal hypercapnia. Because carbon dioxide is a very effective chemical, a minimal increase of its concentration can effectively influence the metabolism of the organism. The elevated carbon dioxide level may be one of the causes of psychosomatic disorders. It is well known that anger through high $FetCO_2$ is a significant independent predictor of hypertension -- at least in females (Van Diest et al., 2003; Scuteri et al., 2001). An early hypothesis also predicted the importance of anger in pathomechanism of hypertension (Alexander, 1939 as cited in Scuteri et al., 2001). Anger itself influences the development of cardiovascular diseases (Eng et al., 2003). A person having type D personality is predisposed to several organic diseases, e.g. to coronary heart disease (Mols & Denollet, 2010). According to Pedersen & Denollet (2003) type D personality predicts increased risk for cardiovascular morbidity and mortality (odds ratios are from 4.1-8.9). High $FetCO_2$ probably also plays part in the pathomechanism of type D personality, since anger is a feature of type D personality.

5. Panic disorder, hyperventilation syndrome, generalized anxiety disorder, depression

If we accept Andrews' et al. (1990) hypothesis, that there is only one "general neurotic syndrome", we have to demonstrate the main differences among the different anxiety disorder syndromes. Undeniably, there is large overlap between the symptoms of hyperventilation syndrome and panic disorder (Cowly & Roy-Byrne, 1987; Han et al., 1997). The somatic symptoms of the two diseases are entirely the same, except for the fact that there is no panic attack in the hyperventilation syndrome. Chronic hypocapnia occurs in both disorders, also there are decreased bicarbonate levels associated with both (Gorman et al., 1985; Papp et al., 1989). The most important difference between the two conditions probably lies in the explicit irregularity of breathing in panic disorder (Wilhelm et al., 2001b; Caldirola et al., 2004); breathing irregularity is not proven to be definitely connected to hyperventilation syndrome.Regular chronic hypocapnia involves compensatory processes, partly via renal, (Gennari et al., 1972), partly via tissue buffer mechanism (Boron, 2004). Intracellular pH may become completely neutralized, in which case the patient has no symptoms. The intracellular pH can be balanced between chronic hypocapnic alkalosis and „compensatory" metabolic acidosis in the case of panic disorder (Sikter et al., 2009). Panic

attacks usually start with a sudden increase of carbon dioxide level up to or slightly above normal levels (Klein, 1993). The sudden increase of carbon dioxide level also evokes acute, robust hypocapnia (via a brainstem reflex) – the panic attack begins definitely in this period (Gorman, 2003). What has happened? We can see it on Fig. 1.

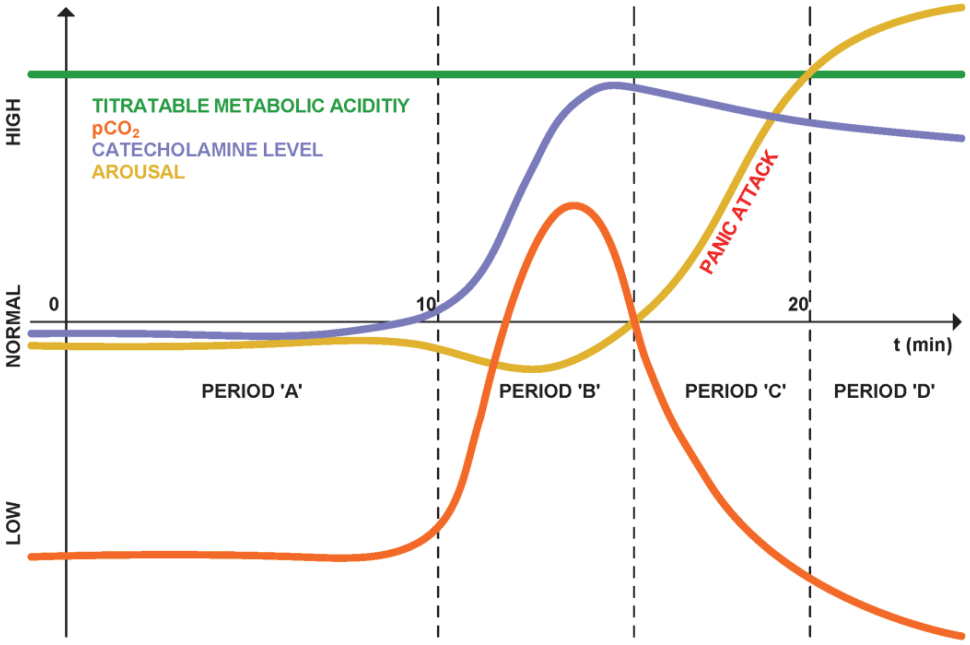

Fig. 1. Schematic diagram of respiratory panic attack, hypothesis (Sikter et al., 2009)

In period 'A' chronic hypocapnic alkalosis balances chronic metabolic acidosis, the intracellular pH is neutral, the arousal response is normal. In period 'B' the CO_2 level starts to increase sharply, intracellular pH becomes acidotic secondary to the high penetrance of CO_2, catecholamine release from different cells increases, e.g. noradrenaline output from locus coeruleus (Filosa et al., 2002) ; the cells' sensitivity to catecholamines decreases and the entire arousal response diminishes slightly. In period 'C' the CO_2 level decreases reflexively, intracellular pH becomes alkalotic, cellular/neuronal responsiveness to catecholamines increases, catecholamine level continues to be elevated, there is ongoing hyperarousal. In period 'D' there is an exaggerated hyperventilation, catecholamine level decreases slowly to normal (the half life of noradrenaline is one or two minutes) (Benedict et al., 1978), although the arousal level remains high for longer because of fear and fright. In this "psychogenic period" (period 'D') pCO_2 might decrease further while catecholamine level may continue to increase (this is the "fight or fright" stress response).

The CO_2 challenge test has been used for decades (Schaeffer, 1958). CO_2 proved far more potent in eliciting the symptoms of anxiety/panic than did voluntary hypocapnia. This fact became a serious point of argument. Believers of the "false suffocation alarm model" (Klein, 1993) argue that hypercapnia, not hypocapnia, is the cause for the panic attack. It was overlooked that the CO_2 challenge test is also biphasic---hypercapnia is followed by

hypocapnia after cessation of exposure (Gorman, 2003). We should notice that there are similarities among the various stress responses, (e.g. the modified acute stress response) (Steen et al., 1988; Bracha, 2004), that of the panic attack (Sikter et al., 2009), and the carbon dioxide challenge test (Gorman, 2003). Gorman pointed out, that panic attack started after CO_2 provocation in the hypocapnic period: "...in panic disorder patients, we have found that elevated cortisol, fear and hypocapnia are intercorrelated in the few minutes before actually experiencing an acute attack." The mechanism is biphasic in all the three types of situations; a hypercapnic period is followed by a hypocapnic one. Chronic hypocapnia is protective against panic attack, as it was stated by Klein (1993), although it is also a precondition for panic attack of the respiratory subtype. On the other hand, acute hypocapnia can evoke panic attack also (Ley, 1985), although only after abruptly elevating the carbon dioxide level. According to authors of this chapter the intra- and extracellular pH is successfully compensated initially, before the attack, but the acute hypercapnic acidosis would eventually be overcompensated and lead to acute hypocapnic alkalosis. The main problem is that the various compensatory mechanisms work on different time scales. Changing of the carbon dioxide level can change in the entire organism in a few seconds while the elimination of catecholamines last for several minutes, and clearing the blood from metabolic (titratable) acidity takes at least one week (Gennari et al., 1972). This is one of the many reasons why there are no perfect compensatory mechanisms.

Researchers who deny the role of hypocapnia in panic attack usually cite studies that didn't find hypocapnia using transcutaneous measurement of pCO_2 (Hibbert & Pilsbury, 1988, 1989; Garssen et al., 1996; as cited in Roth, 2005). However, it is proven that there is a 7-8 min time lag between the end-tidal and transcutaneous measurement of pCO_2, the latter being delays, on the other hand its absolute level is much higher (Wollburg et al., 2009). Often, by the time this parameter is measured, the panic attack is already over. We never stated anyway that all panic attacks would be of respiratory origin.

According to the above described panic theory, the hyperventilation syndrome would differ from panic disorder in that in it the sudden, significant increase of the carbon dioxide level was absent, perhaps secondary to weaker defense mechanisms against hypocapnia in hyperventilation syndrome than in panic disorder. The second possibility may be a consequence of personality type (there is no fear or fright from somatic sensations) or it may be a learned behaviour (e.g. after psychotherapy). Is it possible that with the help of cognitive psychotherapy, and through cessation of psychogenic vicious cycles a patient with panic disorder could be "switched" to hyperventilation syndrome instead? (See Fig. 2.) During period 'C' the signs of sympathicotonia appear, although it is not followed by "psychogenic" period 'D' because of lack of fear. According to our panic model residual symptoms and breathing abnormalities would remain even after cognitive psychotherapy (Meuret et al., 2010), which explains why one commonly finds evidence for the exacerbation of panic disorder during long-term clinical follow ups (Durham et al., 2005).

In the resting state the pCO_2 levels of GAD patients were slightly lower than that of normal controls (Wilhelm et al., 2001b). Their breathing irregularity was milder than that of patients with panic disorder, nevertheless there was disordered breathing. Oscillating pCO_2 levels can evoke higher arousal even when slightly below normal (Bruton & Holgate, 2005). Intracellular pH also interferes with oscillating catecholamine levels and causes fluctuation of arousal. Namely both pCO_2 and catecholamine levels are fluctuating but at different rates – their effects on arousal sometimes add up, sometimes netting a weaker response. Neurons in the brain are working synchronously. Neurons that are linked together are able to become

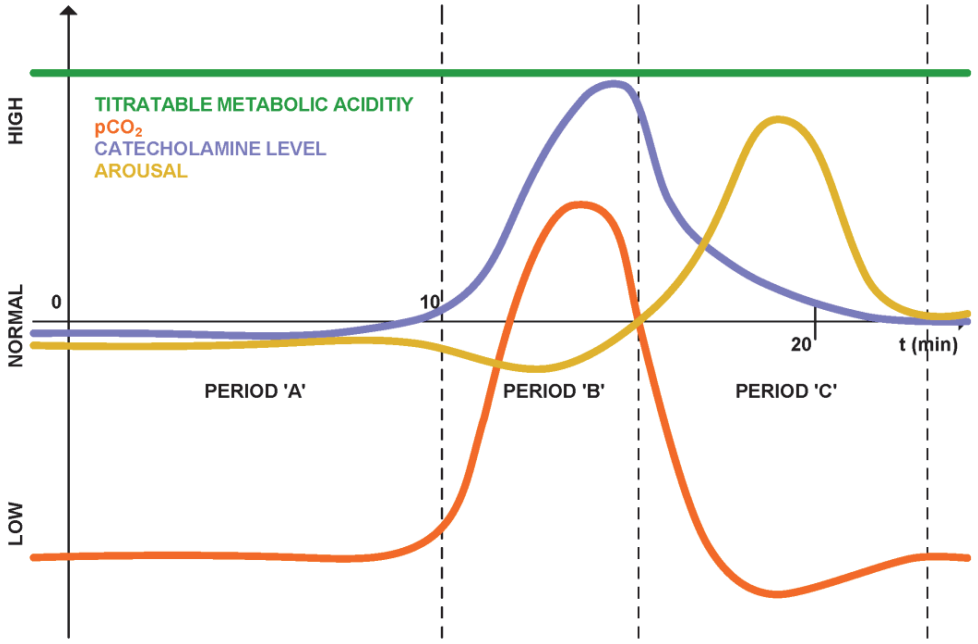

Fig. 2. Schematic diagram of respiratory panic attack after psychotherapy, (hypothesis)

both hypo- and hyperarousal. Positive and negative emotions also affect breathing patterns and arousal (Homma & Masaoka, 2008). "In respiratory patterns, respiratory rate is changed dramatically by emotional changes. It can also be emphasized that changes of respiratory rate are related to individuality." This cavalcade of alterations can make a person psychologically unstable and can initiate vicious cycles through psychogenic mechanisms.

Clinically, anxiety is a hyperarousal, depression is a hypoarousal manifestation of an "internalization disorder" with the participation of the limbic system. This concept is supported by the presence of associated comorbidities. "An estimated 85% of adults with depression experience significant symptoms of anxiety, and 58% have a diagnosable anxiety disorder during their lifetime" (Gorman, 1996/1997; Kessler et al., 1996 as cited in Lenze et al., 2000). Anxiety disorder is linked with dysthymia as well as with major depression. Generalized anxiety disorder is associated with dysthymia much more frequently than with bipolar or unipolar depression (Pini et al., 1997). It seems as if anxiety and depression would be the two end extremes on the spectrum of the same disease entity. Generalized anxiety disorder stands in the middle. As we mentioned above, in GAD pCO_2 levels oscillates around a normal baseline, and its interference with catecholamine levels and emotional tension would alternately result in anxiety or dysthymia. (See above.)

We suggest that every hyperarousal state has hypoarousal periods as well. The pH changes have to be corrected from time to time to maintain homeostasis. (Cytosolic pH is limited to a narrow range even in pathological conditions.) According to the authors' hypothesis there are three ways for the neurons of limbic system to become hypoaroused and thus result in clinical depression. A./Via cytosolic acidosis, e.g. through increasing pCO_2 level. B./Existance of high basal cytosolic Ca^{2+} level in major depression (e.g. in neurons of

hippocampus), which is partially genetically determined. C./Running out of ATP. (Example: intracellular alkalosis increases inward Ca^{2+}-current, this Ca^{2+} overload requires more pumping activity and ATP energy -- the energy supply may become insufficient after a long-lasting alkalosis. The increased Ca^{2+} conductance and good energy supply at the questioned neurons are prerequisites of anxiety, while low Ca^{2+} conductance and ATP deficiency lead to depression.) There are very important differences between dysthymia and major depression. Dysthymia is a functional disorder, it is constantly changes in severity and alternating with anxiety. Major depression (unipolar) is extremely stable; without treatment it result in hippocampal atrophy (Sheline et al., 1996; Videbech & Ravnkilde, 2004). This suggests that it could be an organic disorder. (See: Sick Cell Syndrome).

6. Sick cell syndrome as a manifestation of the Second Law of Thermodynamics

Life needs stability and high-grade permanency, which is only a surface because it is the resultant of two opposite processes, namely of anabolism and catabolism. If the two are in equilibrium, a steady state exists. The life seems to contradict the Second Law of Thermodynamics, because cell/organism exist persistantly on higher energy level than its surround. It looks as if the Second Law of Thermodynamics could not prevail in biology. It was said dogmatically in the 20th century, that is because the „life (cell/organism) is an open system". Though it has proven for the last two decades, that the living cell/organism is not totally and not always open for its surround (Hayflick, 2007), the Second Law of Thermodynamics can prevail tendentiously also in biology. It cannot be otherwise, because plenty of incidents would not be explicable. How might be, that nearly all well known illness is linked to magnesium deficiency and calcium accumulation in the cells? How might be, that our force gradually decreases after 25 year-old, and it does not increase by chance for a longer period? How might be, that everybody dies? Etc. The amount of adenosine triphosphate (ATP) is the best index of viability, because cells use this "biological energy" almost exclusively during energy required biochemical procedures. A cell usually dies, if it looses two-thirds of its ATP content (Farber, 1973). Specific ion transport ATP-ases (Na^+,K^+-ATPase, Ca^{2+}-ATPase, H+-ATPase, etc.) "pump" specific ions across cell membranes. It costs a lot of energy (30-45% of basal metabolic rate). Antiporters (e.g. sodium-calcium exchanger) and symporters (e.g. co-transpors) use electrochemical potential difference of ions (in this way using ATP energy indirectly). Every cell has different primary and secondary transporters, which exert to maintain and restore the interior ionmilieu. (According to Claude Bernard, the constant "milieu interieur" would be the guarantee of viability and eternal life.) ATP content of cells decreases with aging and illnesses (Barbagallo et al., 1999). Cells/neurons struggle against equilibration of ions in their whole life, to maintain chemical potential of ions between extra- and intracytosolic space. The authors created a hypothetical equation based on literature data:

$$\frac{[K+]\times[Mg^{2+}]\times[HPO_4^{2-}+H_2PO_4^-]\times[Zn^{2+}]\times[N]}{[Ca^{2+}]\times[Na^+]\times[Cl^-]\times[H^+]} = ATP \qquad (1)$$

According to the Equation 1. the ATP concentration of cell correlates with concentrations of K^+, Mg^{2+}, Zn^{2+} and inorganic phosphate (P_i) (we also name them "cytosolic ions"), and ATP

inversely correlates with the cytosolic concentrations of Ca^{2+}, Na^{+}, Cl^{-}, and H^{+} (we also name them "extracytosolic ions"). Resting membrane potential also correlates with ATP concentration and the quotient of the fraction (Bilbrey et al., 1973). (It was practically speaking earlier, that resting membrane potential is equal with "potassium potential".) It is indirectly proved, that nitrogen (=protein N) content of sick cells also decreases parallel with ATP and electrolytes in the numerator after their long-lasting existence (Wacker & Williams, 1968; Wang et al., 2004). Lack of intracellular K^{+} correlates with Mg^{2+} (Whang et al., 1992), there is also connection between lack of Mg^{2+} and inorganic phosphate (Quamme, 1997). Elevated basal cytosolic Ca^{2+}, decreased Mg^{2+} and ATP are the missing links, which may join several endemic, aging-associated disorders together (Barbagallo et al., 1997, 2009). Intracellular Zn^{2+} content correlates with protein content (Walsh et al., 1994). Relations of K^{+}, Mg^{2+}, Zn^{2+}, inorganic phosphate, and protein of the sick cells are constant, and correlate with ATP content – in the case of steady state. It is hypothetically a universal biological thesis, the consequence of the Second Law of Thermodynamics. We could differentiate the functional disorders (e.g. anxiety disorder) from the organic ones (e.g. delirium in elderly people, organic psychosyndrome), that "sick neurons" of previous patients' brain would contain normal, but of latter would contain decreased amount of protein.

7. The role of ions in anxiety disorder

There is substantial amount of data about magnesium deficiency, excitability of neurons, stress and similar topics (Durlach et al., 1997). The relationship between magnesium deficiency and aging is obvious (Barbagallo et al., 2009). There is also huge amount of data supporting the importance of electrolyte function to the body and the consequences when imbalance occurs. It seems that the electrolyte disturbance belongs to the "disease" itself. To avoid chaos we had to model diseases, the result is the Modified Sick Cell Syndrome. (See above.) Its essence is the followings: The lack of "cytosolic ions" (K^{+}, Mg^{2+}, Zn^{2+} and $HPO_4^{2-}/H_2PO_4^{-}$) develops always together in sick cells in the case of a steady state. Its lack is inseparable from accumulation of "extracytosolic ions" (Na^{+}, Ca^{2+}, H^{+}, and Cl^{-}) in cytosol. The alterations are proportional with decrease of ATP, namely Mg^{2+}: ATP (and also other "cytosolic ions") ratios remain stable in steady state. Resting membrane potential usually decreases also proportionally. (Although these statements are simplifications, reality is surely complex. This model is only a compass.) Usually more ions enter cytosol than leave it. Volume of damaged cells increases at first because of increased osmolality. Protein content of cytosol also starts to degrade – proportionally to ATP and "cytosolic ions" -after a long-lasting disease (see Equation 1.) the cell starts to atrophy. Electrolyte deficiency was rarely investigated parallel intracellularly, authors usually analyse them one at a time. Symptoms caused by potassium deficiency (adynamia, fatigue, tiredness, muscular weakness, emotional and mood instability, apathy, nausea, feeling of metallic taste, tinnitus, hearing problems, meteorism, constipation, orthostatic hypotension, non-characteristic cardiac symptoms; in more severe cases vomiting, areflexia, paraesthesia, tetaniform symptoms, etc.) can be very easily misinterpreted as „neurotic symptoms". Symptoms of experimental, human phosphate deficiency (including weakness, loss of appetite, bone pains, malaise) can also appear like anxiety (Lotz et al., 1968). The main symptoms of zinc deficiency include anorexia and weight loss, which are at the same time frequently occurring functional symptoms (e. g. in anorexia nervosa). The pathological role of zinc deficiency is also well known (Sandstead et al., 2000). Neurons are very sensitive to

magnesium deficiency and even a small deficiency may cause hyperirritability. It is known that anxiety and stress are related to magnesium deficiency (Galland, 1991-1992; Grases et al., 2006). Magnesium (and consequent cytosolic electrolyte)-deficiency is not specific to a certain functional disease (Durlach et al., 1997). Elevation of intracellular proton concentration is a part of Sick Cell Syndrome. However, not only intracellular acidosis, but potassium and/or magnesium and/or phosphate deficiencies can cause hyperventilation, also (Knochel, 1977). At the same time, long-term hypocapnia reduces the concentration of ionized magnesium and causes hypokalemia and hypophosphatemia (Watt et al., 2001). This is also supported by study of Paleologos et al. (2000). Therefore, the possibility of development of a vicious circle is given. Lactate-induced panic reaction was more intensive in those panic disorder patients, where lower serum phosphate levels were measured before administration of lactate (Gorman, 1989). Hypophosphatemia is especially important factor of the Sick Cell Syndrome, because it can generate directly ATP deficiency (Knochel, 1977) in cells according to the Path of Least Resistance (see below).

The effect of long-term of hypocapnia on ions (Okel & Hurst, 1961; Paleologos et al., 2000.; Watt et al., 2001) is especially important since it can help to demonstrate how the Sick Cell Syndrome and the Locus Minoris Resistentiae work. It seems that apart from the abrupt effect of biphasic hyperventilation (see Fig. 1.) there is a second, slightly slower effect of chronic hypocapnia. We have to suppose that chronic hypocapnic alkalosis (after one or two hours) starts to rearrange of "cytosolic ions" in several tissues at least. There is no dramatic alteration in the extracellular space except of hypophosphatemia. On the other hand, "something" would have happened in cells (only after a long-term existance), if hypocapnia was held at a stationary level. This "something" is essentially different in each person. Hypothetesis: ATP deficiency develops in the cells/organs, which are tolerating hypocapnia less, than the others (see: Locus Minoris Resitentiae). Damage will develop in these cells according to the Sick Cell Syndrome with alterations of ions and decreased resting membrane potential of these cells. In the case of existing intracellular alkalosis (e.g. acute hypocapnia) these neurons will be "firing". Distinct people have different Locus Minoris Resistentiae, that is why the symptoms will differ (Okel & Hurst, 1961). In the case of the alteration is moderate and/or not permanent ("functional disorders") there is no significant protein degradation, but in the case of permanent, significant noxious agent (e.g. major depression without treatment) protein degradation and atrophy also develops (Sheline et al., 1996). It seems that „functional" vs. „organic disorder" is only a quantitative question. (See: Treated vs. untreated major depression.) Intracellular Mg^{2+} and bioenergetic deficiency of brain neurons are proved in vivo in major depression (Iosifescu et al., 2008).

The effectiveness of electrolyte (magnesium) supplementation gives the best evidence regarding etiologic role of deficiency in the observed symptoms, since it is very hard to prove technically the intracellular ion deficiency (Durlach et al., 1997). Magnesium supplementation studies of functional diseases bring mostly positive results in depression (Cardoso et al., 2009) and in anxiety symptoms (Lichodziejewska et al., 1997). Abundant potassium intake has a protective role in brain, at least against vascular events (He & MacGregor, 2001). It was found antidepressant effect in animal models and was suggested a potential antidepressant activity of zinc in humans (Koroczka et al., 2001).

Dr. Sikter (as practitioner internist and cardiologist) and his 13 colleagues have treated more than 1000 patients with anxiety disorder during last twenty years with mixture of salts, which contained "cytosolic ions" (K^+, Mg^{2+}, Zn^{2+}, and $HPO_4^{2-}/H_2PO_4^-$) together (see: Disclosure.) The patients typically had cardiac complains (palpitation, breathlessness,

paraesthesia, fatigue and also sighing tendency, which presume hyperventilation). The female/mail ratios were 4/1-5/1. The cardiologists surprisingly experienced that complains disappeared or substantially decreased at more than 80% of the cases after several days (<7days) administering 30-40% of the Recommended Dietary Allowance salt combination. Naturally we have to suppose placebo effect, because there was no placebo control, although the twenty-year experience is too long time to lapse. Both the doses of salts and the time of its action are very surprising, almost unbelievable. It is impossible that these amounts of salts would supplement the supposed lack of ions for several days. It was supposed that the four ions help each other in synergism, the salt mixture should have acted on breathing centre(s) to decelerate breathing. The increasing pCO_2 level could decrease arousal and complains via increasing intracellular H^+ concentration. It is also possible, that hypophosphatemia has bigger importance, than it was thought previously (Gorman, 1989), when phosphate therapy was not tried yet. A placebo controlled study should be done.

8. Locus Minoris Resistentiae

Most of the diseases affect the whole organism to one degree or another. Stress, hypocapnia, electrolyte disturbances also act on the organism. Though, most of the patients have predisposed organ vulnerability to illness (or the illness worsens) from the different noxious substances. Why does a pathogen substance (in our example hypocapnia) cause different complaints? Why do different pathogen substances cause (or worsen) illnesses on the same organ in a given patient? It may be explained with the theory of the Locus Minoris Resistentiae or the Path of Least Resistance. According to the Sick Cell Syndrome theory the illness harms the metabolism of cells, degrades also its energetical basis and interior ionmilieu. In the case of any seriously harmful noxa, which affects on the organism (e.g. hyperacute illness, cancer), it causes catabolism and degrades a part of (cells)-cytoplasm. Anabolic reparation of tissues/cells cannot start until the harmful effect exists. If it stops, cells start to repair themselves, they start to rebuild cytoplasm. Cells build-in ions first into cytoplasm with ATP energy (Jeejeebhoy, 1994). The available electrolytes in the extracellular space usually are not enough to supply „hungry" repairing cells – cells of organism struggle against each other for electrolytes. Those cells having worse metabolism and less ATP content will loose fight, and they remain or become more and more ill. The cells regarded as the Path of Least Resistance are the weakest chain links of the organism. In the case of a weak harmful noxa affects on tissues/cells of the organism (e.g. alkalosis induced by hypocapnia), cells are able to repair themselves continously and fight against the damage. They restore their original ionmilieu, but not completely and not equally in the whole organism. The weakest cells/tissues get the worst of them, and they become ill, at first functionally, then organically. Cells may tolerate damage differently even in the same organ or same tissue by having different kinds of metabolism and different ATP energy contents. That statement is particularly important at organs containing electrically excitable cells (e.g. central nervous system or heart). That means certain cells will become functionally affected (and they start firing frequently or slowly) while other cells will not. That is why a noxious agent (like acute or chronic hypocapnia) can cause different mental, organic or psychosomatic disorders on different patients.

8.1 Psychogenic asthma bronchiale as an example of Locus Minoris Resistentiae

Organic diseases (e.g. organic pulmonary disorders as asthma bronchiale) often cause hyperarousal mental disorders too (Dratcu, 2000). On the other hand, hyperarousal mental

disorders often provoke (or activate) asthma bronchiale attack, which is thought to be sometimes purely psychogenic (Iamandescu & Mihăilescu, 2008). Growing amount of data proves, that hypocapnia causes bronchial spasm and maybe evokes (triggers) asthma bronchiale in patients whose Locus Minoris Resistentiae is their airway smooth muscle cells (Bruton et al., 2005). We agree with Lindeman et al. (1998 as cited in Bruton & Holgate, 2005), "that hypocapnia has a direct effect on airway smooth muscle cells, possibly via the effect of intracellular alkalosis on intracellular free calcium concentration". Responsiveness of airway smooth muscle cells to hypocapnia is extremely different at distinct patients. Hypocapnia (psychogenic stress) can evoke asthma attack in certain patients. The provoked attack may cause also arterial hypoxia and dyspnoea, which increases the hyperventilation, - a vicious cycle. We agree with Bruton & Holgate (2005), that the generally accepted "normal" arterial pressure of carbon dioxide ($PaCO_2$), namely 35-45 mmHg is too wide. The normal $PaCO_2$ is 39-41 mmHg according to Bruton & Holgate (2005). There are data, which prove that 35-38 mmHg $PaCO_2$ may be already in pathological range (Bruton & Holgate, 2005). pCO_2 level changes permanently during the life (we cannot „pin" it), it is not a well reproduceable parameter, the carbon dioxide sensitivity test (Schaeffer, 1958) seems to be a better one.

9. Delirium

It is hard to recognize what the different types of delirium have in common. Delirium is a hyperacute energy (ATP) insufficiency in central nervous system, mainly in cortex. In its background, there are different metabolic and/or organic causes.
Significant hypophosphatemia can cause sudden ATP deficiency in brain (because of the lack of inorganic phosphate in cytosol).

$$ADP + P_i = ATP + H_2O \quad (2)$$

According to Equation 2, cytosolic ATP concentration correlates with cytosolic inorganic phosphate concentration - in steady state. It is very difficult or impossible to differentiate between symptoms of delirium and those symptoms caused by **severe hypophosphatemia** on central nervous system (Knochel, 1977). If the extracellular phosphate concentration is low, the handicapped brain neurons are defeated by other cells/organs. (See Locus Minoris Resistentiae.) According to Equation 1 the membrane potential will also decrease, it gets near to the threshold potential, and will be "firing". The direct cause is often the exaggerated hypocapnia, which decreases serum inorganic phosphate level, cerebral circulation and increases Ca^{2+} conductance of neurons at the same time.
Delirium is observed to develop during incorrect refeeding after long-lasting starvation. (It is so called **„refeeding syndrome"**.) Giving less minerals and more protein to the malnourished, chronically starving patients, severe electrolyte deficiency can develop in the extracellular space as well. In this case the hypophosphatemia is especially dangerous. Acute energy deficiency of central nervous system can appear among symptoms of delirium. (But patients did not drink alcohol at all.)
Delirium in „refeeding syndrome" is the key to other types of delirium. After chronic alcohol abuse **delirium tremens** frequently develops after alcohol withdrawal. Alcohol continuously poisons cells of the organism in the case of chronic alcoholism, but it maintains a pathological balance. After abrupt withdrawal of alcohol the balance falls over. The cells of organism start to regenerate, but the „cytoplasm building minerals" are missing, because

they left the organism during catabolic, poisoned state through kidneys. The developing serious hypophosphatemia can cause acute energy deficiency mainly in central nervous system. Acute hypocapnia, which is an obligatory symptom of delirium tremens, decreases cerebral circulation and O_2 supply, and it also increases energy demand and causes high arousal. It is not well known the connection between hypophosphatemia and delirium tremens, (that is because serum inorganic phosphate test is not a routine examination). However, incidence of hypophosphatemia is 30-50% among hospitalized alcoholics (Funabiki et al., 1998). Hypocapnia is the most common cause of hypophosphatemia in hospitalized patients (Ratkovic-Gusic et al., 2004). There is inverse correlation between pCO_2 level and hyperarousal symptoms of brain during alcohol withdrawal (Victor, 1977). Chronic alcohol intoxication is one of the best documented manifestations of the Sick Cell Syndrome.

The structure and pathology of anxiety dramatically alter with aging. This may be because (according to the Second Law of Thermodynamics) cytosol has a tendency to increase H^+ and Ca^{2+} level with aging. Both of them decrease the Ca^{2+} conductance and arousal. On the other hand resting membrane potential also decreases during this process, and it gets near to threshold potential. Depolarisation of the neurons can develop suddenly at a critical value of resting membrane potential. When the Ca^{2+} conductance increases, arousal can arise suddenly. That is, the pathomechanism of arousal in elderly is often different from arousal in youth. Delirium often occurs among demented patients too. We suppose that the pathomechanism of delirium developing in demented patients is similar to aforesaid, although we did not find any data in relation to hypophosphatemia or hyperventilation regarding **delirium in elderly**. Miyamoto et al., (2001) created an animal model of „postoperative delirium in elderly". **Postoperative delirium** develops among those groups of elderly patients, with whom it might occur spontaneously as well. It is supposed that pathomechanism of both postoperative and elderly patients' delirium are similar. Precondition of developing delirium is pre-existing damaged brain or significant cerebrovascular insufficiency. Damaged, sick cells usually have a lower resting membrane voltage potential. The threshold potential gets closer to resting potential, that is why the damaged cells are often more excitable. Hyperventilation/hypocapnia plays an essential role also in the cases of postoperative delirium under mechanical ventilation. Patients that are mechanically hyperventilated keep on overbreathing for a while even after the operation that is why they go into delirium.

Delirium developing after hospitalization might be caused by Cannonian "fight or flight" response, because patients having damaged brain did not perceive the situation properly and they suppose to be in danger. Feeling horror they can release enormous amount of catecholamines and start hyperventilating. We suppose vicious cycle develops in the cases of spontaneously evolving delirium in elderly. Patients with damaged brain tend to get involved in hyperventilation and delirium – frequent delirium causes hypoperfusion of brain and harms it, causing more brain damage.

Because hypocapnia and lack of "cytosolic ions" play important role in the pathomechanism of delirium, both carbon dioxide and electrolyte therapy might be effective.

10. Breathing retraining

Based on the current literature, breathing retraining is an early curing method for anxiety disorder and asthma bronchiale, but its exact mechanism of action is not known (Courtney,

2008). In 1952, Buteyko theorized that "hidden" hyperventilation is the basic cause of asthma (Bruton & Holgate, 2005). Buteyko worked out a unique breathing therapy with breath control (without $FetCO_2$ control). Millions used his method. It was said that it "could cure a large number of the chronic ailments affecting modern society" (Courtney, 2008). It is excluded that using Buteyko's method restores the normal carbon dioxide level, although it may be useful also in anxiety disorders. We should suppose that it stabilizes breathing and decreases carbon dioxide fluctuation (Courtney, 2008), but without controlling $FetCO_2$ the efficiency breathing retraining is fortuitous (Meuret et al., 2008). In fact, it can be even harmful. Therapists having no equipment usually exert to make breathing of their patients steady. If they succeed to maintain breathing steady, the treatment may be partly successful. Arousal decreases, anxiety pauses, if the pCO_2 level does not fluctuate. Although without equipment to monitor pCO_2, this goal is often unrealistic. On the other hand, in the state of chronic hypocapnia, control mechanisms of organism can try to restore normal carbon dioxide level, which could involve irregular breathing and arousal (see panic attack). Even though it is plausible to use capnography-assisted respiratory training in the cases where chronic hypocapnia evidently exists (e.g. in panic disorder), psychiatrists start to apply this curing method only nowadays (Meuret et al., 2008; Meuret et al., 2010). Ninety-six percent of patients achieved "much" or "very much improvement" at 12-month follow-up. "…the 68% panic-free rate a12-month follow-up do suggest that this is a potentially potent therapy that warrants more definitive testing" (Meuret et al., 2008). It seems the authors were also surprised at their results, and started to underestimate them… "…We are uncertain whether the physiological correlates of anxious states that we have observed are markers, causes or simply concomitants" (Roth, 2005).

The authors of this chapter think that it is the time to end doubting about harmful force of alternating carbon dioxide levels and to undertake this issue with exquisite conviction similarly as Donald Ley and Donald Klein have done before (Ley, 1985; Klein, 1993). There is now the technology available to assess dysfunctional habitual breathing patterns in real time and in a quantifiable way. Despite this fact the thousands of therapists and trainers teach people how to breathe correctly, we found only one scientific publication on PUBMED (Meuret, et al., 2008) describing the importance of breathing regularity and its physiological implications. It would be considered common standard practice to assess the breathing pattern since its importance is well documented in orthodox medical literature. One of the authors (De Guevara) is a breathing trainer, and his experiences are the follows:

Participants are clients who are seeking alternatives to their symptoms such as anxiety, fatigue, nervousness, migraines, headaches, and unexplained chest pains. Common complaints include breathlessness, agitation, lack of attention or poor focus ability, "out of control and out of balance" feelings. The client has undergone the necessary medical tests to rule out major organic diseases.

Feedback mechanism of actual breathing patterns and pCO_2 monitoring sets the stage for the client to learn how to regulate and maintain homeostatic breathing in all contexts whether relaxed or stressed, active or passive. The client's goal is to achieve pCO2 levels ranging from 38-43 mm Hg in the end-tidal value ultimately without the use of the instrumentation. The role of the breath trainer is director at first, but later facilitator. The client learns to interpret the changes in breathing patterns, changes in carbon dioxide levels, and somatic sensations experienced in the process. Reliance on the capnograph is encouraged at first, but discouraged towards the second half of the training.

Training focus and objective are centered on learning and self-exploration rather than prescriptive exercises based on treatment recommendations. Emphasis on normalizing pCO2 levels rather than respiratory rate. Respiratory rate, locus of breath (diaphragmatic or thoracic), are addressed in the beginning of the training, but are not the ultimate frontier. Diaphragmatic breathing does not guarantee optimal respiration. Breathing training for performance addresses embraces negative play or erratic breathing as part of the methodology. It is equally important for the client to be mindful of unstable versus stable breathing. pCO2 monitoring in different breathing rates while maintaining pCO2 levels in the desires range is only possible by capnography.

Restoring the breathing patterns is more of an art than a science. People learn differently and different needs require different strategies. It is about undoing patterns of unstable breathing. The body struggles to return to the old deregulated patterns. It was thought that these breathing patterns are intimately connected with memories, daily activities, emotionally charged experiences, and even leisure. The authors think that the altered pathological breathing pattern is also linked to altered interior ion-milieu of breathing centres, which helps to maintain the permanent hypocapnic state. Meanwhile the client starts to restore his/her euventilation kidneys clear organism from needless, noxious ions and retract the missing salts. It takes 5-7 days at least, but several weeks or months.

For the chronic over-breather where the pCO2 levels are below 37 mm Hg, reaching 40 mmHg gives them a sense of euphoria and relaxation. This is only experienced the first time they reached 40 mm Hg in the initial session. After this, they seem to maintain focus, calmness and centeredness as a new inherent pattern that is unconsciously done. Other clients report feeling very sleeping when reaching 40 mm Hg during the first session, but with sustained training, their energy levels improve dramatically and it is sustained consistently throughout the day. They experience an overall sense of well-being by improved stamina. The client who successfully completes the program reports increased focus capacity and decrease emotional reactivity in stressful events where confrontation with others is inevitable.

For some clients, changing breathing patterns and increasing the PCO2 is a struggle. They report of an unnatural way of breathing when increasing pCO2 levels. This is because the body is used to operating in a deregulated breathing pattern chronically. With continuous training and pCO2 monitoring, this feeling is overcame, and a more natural and easy breathing pattern with optimal pCO2 levels are achieved.

Evidently, many people could benefit from breathing training, but not all people will qualify for it. Commitment, desire to change, improve quality of life, and self-determination are traits that play a pivotal role to achieve successful outcomes. The financial commitment has been synonymous with successful outcomes in this training program. The client who successfully completes the program reports increased focus capacity, increased energy, increased relaxation, better sleep patterns, and decrease emotional reactivity in stressful events where confrontation with others is inevitable. For some clients, changing breathing patterns and increasing the pCO_2 is a struggle. They report of an unnatural way of breathing when increasing pCO_2 levels. We suppose that the altered intracellular ionmilieu of breathing centers is what hinders the restoration of eucapnia (see above). Only a small percentage of people do not reach successful outcomes.

In the final analysis, anxiety, whether physiological or psychological, seems to disappear when 40 mm Hg is reached and maintained. In contrast, in other cases, by lowering pCO_2

levels below 28 mm Hg. On healthy young individuals with no previous history of anxiety, anxiety symptoms were created along with other symptoms such as nervousness, irritability or "feeling on the edge", cold extremities, sweaty palms, tingling in the hands and palpitations.
Psychological triggers can cause anxiety state. Commonly, the current literature supports that hyperventilation is the result of anxiety state. Is it possible that anxiety can be caused by habitual unstable breathing patterns? Can behavioural hypocapnia cause a disruption in the biochemistry and phyisiology? Can said changes mediate symptoms that are difficult to explain by standard medical tests? Paradoxically, the medical literature from various specialty areas in medicine that supports the aforementioned abounds. Breathing in itself is the last sought and most underappreciated of all human activities because it is thought to be involuntary and natural. The author's (De Guevara) premise is that a person can learn to disrupt the brain stem chemoreflex by voluntary and habitual deregulated breathing patterns. Breathing is also behaviour, and like any behaviour is subject to learning, emotionally charged experiences, physical injuries, and even leisure activities like in sports or performing arts. Anxiety disappears at 40 mm Hg in the end tidal carbon dioxide as long the participant or breathing trainee can sustain and apply the tools learned in the program. Cognitive behavioural psychotherapy is fundamental and beneficial to help people with coping strategies and self discovery of solutions to their problems. Breathing, in the context of acid-base balance, deserves more attention and recognition. This paradigm may seem to be unknown yet, but many people have experienced relief or eradication of anxiety state by learning how to self-regulate carbon dioxide levels. Participant in breathing training have learned a tool which empowers them with the knowledge and the skills to make themselves well and to take the different dynamics and stressors of life more effectively.
The authors of this chapter hypothesize that capnography-assisted breathing training helps to restore healthy interior ionmileu of cells/neurons. The curing process, the patients' capability to maintain normocapnia could be helped by administering salts containing "cytosolic ions" together, but the two methods were never tested together.

11. Conclusion

There is abundant amount of scientific data regarding the topic discussed in this chapter, but the freshes are not always the best data. To avoid chaos the authors made a believable dynamic model of arousal and anxiety on the cellular level. They posed very important and hard questions about related to the mechanism of anxiety and the majority of questions were answered. They brought up crucial arguments for the role of hypocapnia in anxiety disorder. Brain physiologists proved the importance of intracellular pH in the responsiveness and excitability of neurons. The organism tries to maintain the stability of cytosolic pH and ion milieu, but the various compensatory mechanisms can lead to disregulation and to different disorders as these differ from the original physiological processes. The different mechanisms have time discrepancies. (See proposed hypothetical mechanism of panic disorder.) The authors made an attempt to integrate the main theories of anxiety disorder. They pointed out, that in humans both acute stress response and panic attack are biphasic phenomena. First, hypercapnia elevates the catecholamine level; the second phase hypocapnia renders neurons hypersensitive to catecholamines. The classical carbon dioxide challenge test is designes to simulate this biphasic response: first the carbon dioxide challenge, after which the patient is moved to fresh air where he/she

hyperventilates. It is well known, that patients with anxiety disorder usually have irregular breathing patterns and changing carbon dioxide levels (altering cytosolic pH), which results in fluctuating catecholamine and cortisol levels. The parameters fluctuate in their own rhythms, and interfere with each other and also with the fluctuating psychological tension.
The continually altering arousal can provoke anxiety disorder through psychogenic pathomechanism. (Increased arousal conditions and triggers differents parts of the brain, but it does not lead categorically to anxiety. See Fig. 2.)
The authors propose another model besides this "interference anxiety model". It is a fact that voluntary (or mechanical) hypocapnia provokes symptoms hours to days after the onset. Symptoms can be explained with the concept of the Modified Sick Cell Syndrome and the newly interpreted Path of Least Resistance concept that can explain how and why clinical symptoms would appear only after prolonged hypocapnia (meanwhile the pCO_2 level remains on a similarly low level). Namely after a long-lasting struggle, the cells loose the "struggle for ions" according to the Path of Least Resistance and their interior ionmileu changes; they go into lower energy level with less ATP content and less „cytosolic ions", their resting membrane potential decreases, and these cells become irritable. The authors present the differences of pathomechanisms between hyperarousal in „functional anxiety disorder" and the hyperarousal observed in the delirium of various diseases and states. (They pointed out, that exaggerated hyperventilation, hypocapnia, hypophosphatemia, and ATP-deficiency of the central nervous system are the keywords in all types of delirium, which is not evident in the available literature.) The authors also define the differences between „functional" and „organic" diseases according to the Modified Sick Cell Syndrome theory, and explain that the difference is mainly quantitative.
Exertion correlates with actual arousal in the case of wild animals, because they behave instinctively, while the essence of "civilized behaviour" is to hide arousal, emotions, and instincts. Thus arousal and motion usually do not correlate in humans, which is the main cause of diseases of civilization, the authors state.
According to a new direction there is an important link between personality and hypocapnic/hypercapnic tendency of breathing, although the connection was found to be weak. pCO_2 ($FetCO_2$) is not a well reproducible parameter. Moreover, its "normal range" is too wide. Therefore, the authors propose systematic population surveys regarding breathing patterns, responsiveness to CO_2 challenge, and to standardize personality types. It could help to understand psychosomatic disorders even better.
The authors argue that CO_2 tension is one of the most important "milieu interieur" participants, and it cannot be substituted by any acid components. The ensuing "compensation" for the acidic pH (by metabolic components) does not mean that the homeostasis has been restored. The new steady state is similarly stable as the original was and it hinders the restoration of the original pCO_2 level. Without restoring the original interior ion-milieu the organism will malfunction.
It is suggested that in patients with anxiety disorder one could achieve "restitutio ad integrum" with combination of prescribing salt supplements containing "cytosolic ions", and of psychotherapy (with the resulting cessation of psychogenic vicious cycles), and by addressing the exchange of oxygen and carbon dioxide gases through real time breathing training using capnography. All methods have advantages, but the aforementioned combined therapy would be the most appropriate approach. Psychotherapy is often causal therapy as well, but with psychotherapy alone breathing abnormalities can perpetuate, anxiety can exacerbate, unawares to the individual. The electrolyte therapy is simple, but

there are no placebo-controlled trials. Although still in "child's shoes", capnography-assisted breathing training would be necessary to provide tangible objective monitoring and to achieve better outcomes.

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13. Nomenclature

ATP = adenosine triphosphate
DSM III = Diagnostic and Statistical Manual of Mental Disorders III is published by the American Psychiatric Association in 1980
$FetCO_2$ = resting end-tidal fractional concentration of carbon dioxide
GAD = generalized anxiety disorder
Locus Minoris Resistentiae = Path of Least Resistance
milieu interieur = interior milieu
$PaCO_2$ = partial pressure of carbon dioxide in arterial blood
pCO_2 = partial pressure of carbone dioxide
restitutio ad integrum = restoration to original condition
vicious circle = vicious cycle

14. Disclosure

The treatment of patients (mentioned in subchapter 7.) was carried out according to Dr. Sikter's United States Patent 5348749. (Sikter's patent has been valid since May 1992).
Dr. Sikter declares that this book chapter was not sponsored by anyone.

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2

Relationship Between Oxidative Stress and Anxiety: Emerging Role of Antioxidants Within Therapeutic or Preventive Approaches

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1. Introduction

Oxidative stress (OS) represents a loss of balance in oxidation-reduction reactions (redox state). It is characterized by the reduced ability of the antioxidant defense system to efficiently eliminate the excess of the oxygen-derived species production, eliciting the toxicity of oxygen and its detrimental effects.

OS state is related to probably more than 100 human diseases including male infertility, autoimmune diseases, atherosclerosis, cardiovascular troubles, diabetes and cancer. Interestingly, certain diseases associated with OS disturbances such as neurodegenerative diseases and neuropsychiatric diseases including schizophrenia and some forms of behaviour, such as aggressiveness, depression and anxiety are more specific for the nervous system impairments. In particular, recent research observed a close relationship between OS and anxiety in both human patients suffering from anxiety disorder (obsessive–compulsive disorder and panic disorder), and humans and animals displaying high trait anxiety (Bouayed & Bohn, 2010; Bouayed, 2010; Bouayed et al., 2009). It has been debated that brain OS disturbances might be a plausible pathogenesis and risk factor for several specific diseases of the nervous system including behavioural troubles and disorders (Ng et al., 2008; Bouayed, 2010; Bouayed et al., 2009).

Brain controls several activities and functions including emotion; a correct function of the nervous system, that is mediated by neurotransmitters and its adequate interaction with other regulatory systems including the endocrine system (involving hormones) and the immune system (involving cytokines) is crucial for health. Oxygen is very essential for brain activity and in this respect mammal brain is highly sensitive to oxygen deprivation and even short duration of hypoxia can cause irreversible damage or even death. Albeit oxygen is vital, high oxygen-derived species production (through respiration and metabolism) produces toxicity in brain, owing to its intrinsic oxidative vulnerability (Mariani et al., 2005;

Halliwell, 2006; Ng et al., 2008; Bouayed et al., 2009). Interestingly, human brain uses about 20% of oxygen consumed by the body even though this organ constitutes only about 2% of the body weight (Halliwell, 2006; Bouayed et al., 2009). The high energy needed by the brain may explain its high oxygen uptake. However, the antioxidative defense system of the brain is not well equipped (due to relatively low levels of certain antioxidant enzymes, particularly catalase) to deal with the high rate of reactive oxygen species (ROS) resulting from the brain's high oxygen consumption (Mariani et al., 2005; Halliwell, 2006). Catalase and glutathione peroxidase play a key role in the catabolism of hydrogen peroxide (H_2O_2) into water and oxygen, avoiding the formation of the highly reactive hydroxyl radicals ($OH^{\bullet}$) following the Fenton reaction

$$(H_2O_2 + Fe^{2+} \text{ or } (Cu^{+}) \longrightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+} \text{ or } (Cu^{2+})) \text{ or}$$

Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 \longrightarrow {}^{\bullet}OH + {}^{-}OH + O_2$) (Valko et al., 2007). Brain metabolism produces a lot of H_2O_2, while low levels of catalase have been reported in the most brain regions (Halliwell, 2006). In addition, the abundant presence of iron and copper ions in neuronal cells (Hovatta et al., 2010) may catalyze the production of $OH^{\bullet}$ and $RO^{\bullet}$ generated via peroxides (H_2O_2 and ROOH, respectively). Neuronal membrane lipids, which are rich in highly polyunsaturated fatty acids, may undergo rapid lipid peroxidation following the occurrence of OS (Mariani et al., 2005; Halliwell, 2006; Bouayed et al., 2009). The sensitivity of brain to oxygen increases with increasing age following the natural consequence of brain aging, which is characterized among others by the declining ability of the antioxidant system to prevent against oxidative damages resulting from non-detoxified ROS (Mariani et al., 2005; Pandey & Rizvi, 2010).

The present chapter focuses on the link between OS and anxiety, discussing and reviewing different findings obtained from humans and rodents in this field. The emerging role of antioxidants as a potential new strategy for the prevention and treatment of anxiety is also debated.

2. Anxiety disorder and OS

GABAergic and serotoninergic systems are considered among the principal regulatory systems of anxiety. However, following to recent findings of Kuloglu et al. (2002a and 2002b)— emphasizing that patients with anxiety disorders (obsessive–compulsive disorder and panic disorder), compared to healthy controls, have higher activity levels of the antioxidant enzymes superoxide dismutase and glutathione peroxidase as well as higher lipid peroxidation activity — oxidative metabolism is being also regarded as a plausible pathway that can affect the regulation of anxiety. This hypothesis has gained interest due to the intrinsic oxidative vulnerability of the brain (see above). When the production of ROS prevails over the brain defense systems, the lipid-rich constitution of brain may favour lipid peroxidation, constituting a free radical chain reaction that may result in decrease in membrane fluidity and damage in membrane proteins inactivating receptors, enzymes and ion channels, even disrupting membrane integrity resulting eventually in cell death. In addition to oxidative damage of neuronal membrane lipids and proteins, oxidation of other sensitive components such as nucleic acids and neurotransmitters can occur. As a result, OS can alter neurotransmission, neuronal function and overall brain activity (Bouayed et al., 2009). Therefore, brain oxidative damage might be also a plausible pathogenic factor for

certain multifactorial neurological diseases including neuropsychiatric troubles. Interestingly, OS state was recently linked to other behavioural disorders, such as aggressive behaviour and depression, and also to deterioration of short-term spatial memory (Bouayed et al., 2009, 2010; Dean et al., 2009; Bouayed, 2010), highlighting that OS disturbances could be implicated in the pathophysiology of conditions that are more specific for the nervous system impairment. In this respect, we have reported a statistically significant positive correlation between aggressive behaviour in the resident/intruder test and cell oxidative status in adult male mice (Rammal et al., 2010b). Moreover, patients suffering from major depression have presented OS in both their peripheral as well as their central systems (Bilici et al., 2001, Michel et al., 2007). Curiously, some of these conditions (e.g. aggressiveness and depressed mood) could also be associated with anxiety. For instance, Bayani et al. (2007) demonstrated that in teachers, the high level of anxiety is associated with a high level of hostility. In animals, it has been shown that dominant rodents had high levels of anxiety and they often exhibited aggressive behaviour toward subaltern subjects (Ferrari et al., 1998; Blanchard et al., 1993).

Anxiety may also coexist with depression and for defining these states the term comorbidity is usually used. Such mixed states of anxiety and depression make coping of the disease more difficult. It concerns both general prognosis, as well as treatment response in this cohort of patients. For example, in the Finnish population-wide Health 2000 Survey, it has been estimated that 35.9% of the anxiety disorder patients had a comorbid depressive disorder (major depressive disorder and/or dysthymia) (Pirkola et al., 2005).

In their studies, Kuloglu et al. (2002a and 2002b) have used the activity of antioxidant enzymes (e.g. superoxide dismutase and glutathione peroxidase) in erythrocytes and the level of malondialdehyde in plasma as markers of oxidative status of human subjects (healthy volunteers versus patients suffering from obsessive–compulsive disorder and panic disorder). Other human studies have shown the validity of these biomarkers to assess OS state. For example, several studies have demonstrated that peripheral oxidative status markers in human erythrocytes and plasma significantly correlated with human age, and as a result they have been proposed as biomarkers of the aging process, which is characterized by an increase of oxidative stress with age (Pandey & Rizvi, 2010).

3. High anxiety level and OS

In the light of results from Kuloglu et al (2002a and 2002b) establishing a relationship between OS and anxiety disorder, other recent studies have focused on the link between redox status and normal anxiety, and also on a possible causal relationship between cellular oxidative stress and emotional stress using rodents as animal model. Mice and rats are often used as translational models for studying anxiety in humans due to the similarity in the extremely complex mechanisms involved in anxiety in these species. Indeed, the principal brain areas (e.g. the amygdala) implicated in the processing (or the suppression) of fear and anxiety, the comparable brain circuits involved with anxiety, and the similar neurochemical substrates (e.g. GABA, serotonin) among others, make rodents a good model to study anxiety in humans (Mathew et al., 2008). Anxiety of rodents is detected from specific behavioural model tests, among which the elevated plus maze, the light/dark choice test, open field test and hold board test are most employed. These behavioural tests are also sensitive to pharmacological agents with anxiolytic or anxiogenic properties, causing a decrease or an increase in the anxiety-related behaviour of animals, respectively.

Based on their anxiety-like behaviour assessed in both the dark/light choice test and the open field test, Hovatta et al., (2005) ranked six inbred mouse strains from the less anxious to the more anxious. Afterwards, they have demonstrated a close correlation between brain expression of genes of the antioxidative defense system (*glutathione reductase 1* and *glyoxalase 1*) and anxiety-related phenotypes across all mouse strains. They further found that the activity of the antioxidative enzymes of glutathione reductase 1 and glyoxalase 1 is highest in the most anxious strain and lowest in the least anxious strains. A link between OS and emotional stress is not surprising per se; since it is well accepted that oxidative damage in the brain may cause an impairment of the nervous system. For example, abnormalities in the regulatory systems of anxiety in rodents (e.g. GABAergic and serotoninergic systems) can result in anxious behaviour. Furthermore, alteration of the function of the hypothalamic-pituitary-adrenal (HPA) axis, which is implicated in stress responses and anxiety disorders, could also impact the emotional response. However, the second part of results of Hovatta et al. (2005) obtained from a genetic manipulation using lentivirus-mediated gene transfer was surprising because the role of oxidation or reactive oxygen species is not clear in the genesis of anxiety, despite the role of *glyoxalase 1* and *glutathione reductase 1* in the regulation of anxiety in transgenic mice was established. Indeed, they found that local overexpression of *glutathione reductase 1* and *glyoxalase 1* in the cingulated cortex of the murine brain results in an increase of anxiety-like behaviour, while inhibition of *glyoxalase 1* expression produces low-anxiety. Thus, Hovatta et al. (2005) were able to make a causal link between the antioxidative status of the brain and anxiety-related behaviour supposing that *glyoxalase 1* and *glutathione reductase 1* regulate anxiety in mice. However, *in vivo*, antioxidant genes (e.g. *superoxide dismutase, glutathione peroxidase* and *glutathione reductase*) are normally overexpressed in response to an uncontrolled production of ROS. In certain cases, OS leads to silencing of genes encoding antioxidant defensive enzymes. In the lentivirus experiments of Hovatta et al., (2005) however, the overexpression of the transgenes (*glyoxalase 1* and *glutathione reductase 1*) was induced *in vivo* with a lentiviral vector and not an excessive production of toxic oxygen metabolites. Clearly, the mechanism by which these antioxidant defensive enzymes regulate anxiety is of great interest. Additionally, Hovatta et al., (2005) used *glyoxalase 1*, which is an enzyme of the glyoxalase system, as a marker of oxidative stress, however the link is indirect. Enzymatic activity of glyoxalase 1 aims to protect against carbonyl stress (resulting from excessive accumulation of reactive dicarbonyl compounds). Carbonyl stress leads to protein and nucleotide damages by dicarbonyl glycation, which is associated with several pathologies including diabetes (Thornalley, 2006a, 2006b and 2006c). Glutathione (GSH), which is a major antioxidant in the brain, constitutes a determinant cofactor for the enzymatic reaction that is catalyzed by glyoxalase 1. However, a close relation between oxidative stress and carbonyl stress was established.

Curiously, other findings from another laboratory (Krömer et al., 2005; Ditzen et al., 2006) have complicated the understanding the role of glyoxalase 1 in trait anxiety because they are discordant with those of Hovatta et al. (2005). Contradictory, they have proposed that the level of expression of glyoxalase 1 could be used as a physiological marker of trait anxiety level, with high protein expression indicating low trait anxiety level and low expression for high trait anxiety. Indeed, comparing two Swiss CD1 mouse lines with extremes in trait anxiety, these authors found that glyoxalase 1 was more expressed in the line with low-anxiety-related behavioural phenotype than in the line with high-anxiety-related behavioural phenotype. The expression of glyoxalase 1 has been assessed in several brain areas

and also in red blood cells of mice. The lines of mice with contrasting anxiety-like behavioural phenotypes were generated from wild type mice after > 15 generations of selection. Differences in the genotype of this strain and those used by Hovatta et al. (2005) could play a role in the differences of observed results. Thus, it would be interesting to compare in the same strain, anxiety-related behaviour of mice with their oxidative status rather than compare the redox status of strains differing in their anxiety-related phenotypes. This approach takes into account the intra-variability between individuals of the same strain.

Because of the large heterogeneity in their anxiety levels, Swiss albino male mice (OF1) constitute an interesting behavioural model to study the link between oxidative status and anxiety-related behaviour. Correlation analyses indicated a linear and significant relationship between the intracellular redox status of peripheral blood granulocytes and different parameters of anxiety-related behaviour, assessed in the behavioural light/dark choice test, including latency time (R^2=0.74, P<0.001), cumulative time spent in the lit box (R^2=0.61, P<0.01) and number of entries into the lit box (R^2=0.66, P<0.01) (Bouayed et al., 2007, 2009). Our results suggested a positive relationship between peripheral oxidative status and level of anxiety in mice. To confirm the relationship between OS and emotional stress, we comparatively evaluated the peripheral oxidative status of mice with contrasting levels of anxiety (anxious and non-anxious). Following strict selection criteria from a general population of 100 mice (Rammal et al., 2008a, 2008b, 2010), only 10% of mice were considered as anxious (n = 10) and 10% as non-anxious (n = 10). Thus, mice with intermediate behaviours were eliminated (n=80). We found that high anxiety level was associated with a significant generation of ROS in the peripheral blood lymphocytes, granulocytes and monocytes in mice compared to low anxiety level (Rammal et al., 2008a). Our results confirm that there is a relationship between the level of intracellular ROS in peripheral blood cells and anxiety-related behaviour in mice. These results prompted us to study the oxidative status of the brain in mice with distinct levels of anxiety. Using the same behavioural approach to distinguish between anxious and non-anxious mice, we found that anxiety levels were associated with the oxidative status in both neuronal and glial cells in the cerebellum and hippocampus, in neurons of the cerebral cortex and in peripheral leucocytes (monocytes, granulocytes and lymphocytes) (Rammal et al., 2008b). Our results clearly indicated the presence of OS in the central and peripheral systems of anxious mice. OS in the brain and blood immune cells could predispose anxious mice to neuroinflammation and neurodegeneration as well as recurrent infections. In another study, we have found that high levels of anxiety inhibited part of the cellular and humoral immune systems by significantly decreasing total lymphocytes numbers (including TCD_4^+ and TCD_8^+) and immunoglobulin (A and E) concentrations, emphasising the vulnerability of anxious mice to infections (Rammal et al., 2010a). We considered that type of anxiety evaluated in mice with contrasting levels of anxiety is a trait-anxiety, for two reasons. First, we have verified that the level of anxiety of anxious and non-anxious mice was stable during time (a period of 15 days). In fact, they did not change their status of anxiety in the light/dark choice test. Secondly, we have also verified that in a general population of mice, the anxious (or non-anxious) mice in the light/dark choice test are usually the anxious (or non-anxious) in the elevated plus maze. We have also found that the general activity, both horizontal (locomotion) and vertical (rears), of anxious mice was significantly lower than of non-anxious mice (unpublished results), which was in keeping with the findings of do-Rego et al. (2007) comparing anxious with non anxious male Swiss albinos CD1 mice. These authors also found that these groups of mice did not significantly differ with regard to their immobility

time, marker of depressive behaviour, in the forced swimming test. In agreement with these results, we have also found that the behaviour of anxious and non-anxious mice did not significantly vary in the tail suspension and forced swimming tests, the well-known predictive tests of depression-related behaviour (unpublished results). From the above, it could be suggested that high trait anxiety level in anxious mice from Swiss albino male mice (OF1) was not associated with depressive symptoms.

The results of our studies are in good concordance with the initial findings of Hovatta et al. (2005) associating OS to high trait anxiety level, but our findings do not permit us to declare a causal relationship between these stresses. In keeping with the animal experiments, the link between OS and human trait anxiety was also determined. Indeed, Yasunari et al. (2006) found a significant relationship between trait anxiety and ROS formation in monocytes of hypertensive individuals.

To study the causal relation between OS and anxiety, Masood et al. (2008) provoked OS by depleting glutathione (GSH) in mice using buthionine-S,R-sulfoximine (BSO), and afterwards they assessed the impact of BSO treatment on the level of anxiety. Surprisingly, BSO-treated mice developed anxious behaviour in several mouse models of anxiety including elevated plus maze, hole-board and open field tests. The NADPH oxidase was suggested to be the principal oxidative pathway responsible for the anxiogenic behaviour following BSO treatment. Depletion of GSH was also reported to cause cognitive impairment (short-term spatial memory disturbances) in rodents as assessed in the Y-maze test (Dean et al., 2009). It is also suggested that GSH might play a role in psychiatric illnesses including schizophrenia and bipolar disorder (Dean et al., 2009). However, despite that GSH is considered as a major antioxidant in aerobic cells functioning as an important cellular redox buffer, GSH depletion can cause other cellular stresses, including nitrosative and carbonyl stresses, as GSH is also an important determinant of the nitrogen and dicarbonyl metabolism. Excessive production of ROS induces oxidative damage of cellular structures; production of reactive nitrogen species triggers nitrosylation reactions, which can alter the structure of proteins to inhibit their normal function; excessive accumulation of reactive dicarbonyl compounds leads to damage of protein and nucleotides by dicarbonyl glycation. Additionally, GSH may also have an additional double role in the central nervous system by acting as a neurotransmitter and neuromodulator, e.g. by regulating the release of other neurotransmitters such as dopamine and gamma-aminobutyric acid (GABA), which is an important regulator of anxiety (Oja et al., 2000). Therefore, the anxiogenic behaviour resulting from depletion of GSH in mice could be independent from oxidative metabolism disturbances generated by BSO treatment. Thus, it is difficult to deduce, from this study, a direct causal relationship between oxidative stress and anxiety.

Other studies have mentioned that OS state could cause anxiogenic behaviour, however the link is indirect. Desrumaux et al. (2005) showed that vitamin E deficiency in the mouse brain significantly causes brain OS, resulting in anxiogenic behaviour without abnormalities in the locomotor performance of the mice. Souza et al. (2007) demonstrated in rats that the consumption of a highly palatable diet enriched with sucrose leads to an obese phenotype, increases protein oxidation in the frontal cortex and induces anxiety-like behaviour in the dark/light choice test without altering locomotion in an open field test. Berry et al. (2007) showed that mice developed anxious behaviour during aging, likely due to the accumulation of oxidative damage, which is a characteristic of the aging process in animals. In addition, Berry et al. (2007) showed that a deletion of the $p66^{Shc}$ longevity gene in mice, which results in lower levels of OS and an extended life span, decreased anxiety-related behaviour.

4. Antioxidants as therapeutic or preventive approaches

At physiologic conditions, antioxidants play a crucial role in maintaining redox homeostasis by maintaining the level of ROS at physiological doses necessary for optimal cellular functioning. Thus, the excess of ROS is neutralized by antioxidants avoiding the oxidation of cellular components and consequently their damage. Exogenous antioxidants complete the antioxidative action of endogenous antioxidants by acting together, e.g. additively or synergistically. The principal source of exogenous antioxidants is our diet. However, diets relatively deficient in antioxidants may favour oxidative stress. Vitamin E, vitamin C, carotenoids, zinc, selenium, and polyphenols (e.g. phenolic acids and flavonoids) constitute the principal dietary antioxidants existing in food. Of course, these antioxidants can be found naturally (e.g. in plant foods or animal products such as eggs and honey), however, other sources can also exist (e.g. supplementation and fortification). Currently, there is increasing evidence that the advantageous effects of antioxidants on health are not only attributed to their antioxidant properties. This is due to the fact that antioxidants can also act e.g. as signalling molecules or as chemopreventive agents by displaying other activities such as anti-inflammatory activity (Bouayed & Bohn, 2010). The effect of dietary antioxidants on the central nervous system has gained interest in the last decades. In this sense, it has been demonstrated that dietary antioxidants can also exhibit cognitive enhancing effect, psychostimulant activity, and antidepressant and anti-anxiety properties. For example, it has been shown that antioxidants (e.g. vitamin C, rutin, caffeic acid and rosmarinic acid) possess antidepressant activity with relatively lower doses (0.1-2 mg/kg) than commonly used antidepressants such as imipramine or fluoxetine, which are active at higher doses (≥ 10 mg/kg) in rodents. The mechanism of action of antioxidants on the central nervous system is not well elucidated, however, it has been demonstrated that rutin exerts its antidepressant activity similarly to conventional antidepressants by increasing the availability of serotonin and noradrenalin in the synaptic cleft (reviewed by Bouayed , 2010). Interestingly, antioxidant effects of conventional antidepressants have been reported in several studies (Atmaca et al., 2004; Réus et al., 2010). The antioxidant effects of anxiolytic treatments with citalopram (also used as an antidepressant) have been emphasized by Atmaca et al. (2004) on patients with social phobia. Polyphenols have also shown their ability to reverse anxiety-related behaviour of rodents. Some polyphenols have a pharmacological profile that suggests a partial agonistic action that may produce the anxiolytic-like effects, but without the side effects such as dependency, which are a feature of full agonists such as benzodiazepines. For example, at 3 mg/kg, apigenin exerts its anxiolytic effect in mice without sedation or myorelaxant effects. However, a 10-fold increase in dosage of this flavonoid produced slight sedative effects. Polyphenols may present a dose-effect response on the central nervous system. Rosmarinic acid at a dose of 2-4 mg/kg exhibited anti-anxiety effects, however, in the same animal model this polyphenol exerted psychostimulant effects at the dose 8 mg/kg. Contrarily to conventional anxiolytics, which only have anti-anxiety effects at relatively low doses (1-5 mg/kg), polyphenols can display anxiolytic effects at a spectrum of doses ranging from 2 to 30mg/kg. For example, chlorogenic acid and (−)-epigallocatechinn gallate (EGCG) are active at 25 mg/kg and 30 mg/kg, respectively. The ability of polyphenols to cross the blood-brain barrier might explain the difference in their active concentration. The intranasal administration of polyphenols in the form of liposomes could be an effective strategy both to facilitate the movement of these substances across the blood-brain barrier and to effectively reduce the

active dose. For example, it has been shown that quercetin, which is a flavonoid having difficulty crossing the blood-brain barrier, that its single intrasanal administration (20 µg) to rats reduced the active oral dose (300 mg/kg) by around 6000 times. Noteworthy, the reduction of anxiety was obtained by the oral administration of quercetin (300 mg/kg) but only after one-week of daily treatment. Therefore, the use of liposomes is a potentially novel strategy which can facilitate the delivery of polyphenols across the blood-brain barrier and also can effectively reduce the active dose (reviewed by Bouayed, 2010).

Although psychopharmacological studies present antioxidants as a potential new strategy for the treatment of anxiety and depression, the use of these substances has to be with caution. Several studies are required to investigate the toxicity of antioxidants at non-nutritional doses. At high doses, it has been discussed that antioxidants could enact deleterious effects on health, acting e.g. as prooxidants. As an example, it has been demonstrated that EGCG at pharmacological doses (30 and 60 mg/kg) abolishes anxiety in mice; at 150 mg/kg however this tea polyphenol caused death to mice (100% mortality) in less than 24 h, presumably due to its high hepatotoxicity noticed above 100 mg/kg. Despite that antioxidants, once outside in their natural matrix or at higher doses could be toxic, they, generally, are safe in plant foods due to their presence at physiological doses and also to their resulting combined effect (reviewed by Bouayed & Bohn, 2010). Therefore, antioxidants from a normal diet could prevent from anxiety development. In this respect, it has been demonstrated that some specific foods prevent aging-accompanying anxiety. Viggiano et al. (2006) demonstrated that anxiety of aged rats fed for 10 weeks with a standard diet supplemented with fresh apple fruits was significantly lower than aged rats fed with the standard diet. The decrease of anxiety was not associated with a change in general activity, however a reduction of OS was also found. Indeed, these authors found that brain superoxide dismutase (SOD) activity of aged rats fed with an apples enriched diet was not different from young animals feed with the standard diet with or without apples, while SOD activity of aged rats fed with the standard diet was significantly elevated. Pitozzi et al. (2010) found that aged rats fed, for one year, with a diet containing olive oil naturally rich in antioxidants have displayed a low anxiety than aged rats fed either with a diet containing olive oil naturally low in antioxidants or with maize oil. Interestingly, the reduction of anxiety was associated with significant decreased glutathione reductase activity and expression in the brain. Chepulis et al. (2009) conducted a study on rats fed *ad libitum* for 52 weeks with a diet that was either sugar-free or contained 7.9% sucrose or 10% honey (which is the equivalent amount of sugar). They found that anxiety of rats fed with the diet supplemented with honey was significantly lower than in the other groups. No information was given on the oxidative status of different groups; however, the antioxidant power of honey has already been stressed in other reports. It has been suggested that the anti-anxiety effect of long intake of apples, olive oil and honey may be attributed to the whole food matrix containing complex mixtures of nutrients and non-nutrients including vitamins, flavonoids, phenolic acids, several carotenoids, and many more acting on a synergistic or additive manner, rather than to specific compounds.

5. Conclusion

Anxiety has a multifactorial origin and can result e.g. from pharmacological treatment with some drugs (e.g. methyl-β-carboline-3-carboxylate), stressful situations (e.g. immobilization stress) or natural conditions (e.g. aging process). Although the link between OS disturbances

and anxiety is not disputed, whether oxidative stress is a side effect resulting from emotional stress, or inversely itself is the pathogenesis factor for this condition remains unclear. Nevertheless, results of Masood et al. (2008) showing that the well known anxiolytic diazepam does not fully reverse the anxiety generated by BSO treatment, can suggest that OS could be one of factors causing anxiety. Nevertheless, diazepam can abolish e.g. restraint stress-induced anxiety, although immobilization stress being a prooxidant. Interestingly, Masood et al. (2008) showed that OS-related anxiety could be reversed in mice after inhibition of NADPH oxidase or phosphodiesterase-2, enzyme that is indirectly implicated in OS mechanisms. Salim et al. (2010) demonstrated that anxiety generated by BSO treatment of rats was reversed either by preventive treatment by antioxidant using tempol, or by moderate treadmill exercise. It has been discussed that moderate physic activity reduces the vulnerability of brain to oxidative stress by increasing the resistance of brain antioxidant system following an adaptation, while acute or intense exercise are prooxidants. The vulnerability of brain to oxidative damages is in line with the theory that anxiety could be generated directly by OS. Antioxidants may constitute a potential treatment when OS is the causal factor in anxiety. In addition, a mixture of antioxidants and anxiolytics could be also a useful treatment in patients with anxiety, since OS is associated with anxiety disorders. It has been demonstrated that vitamin C caused a synergistic antidepressant-like effect with conventional antidepressants administered at subeffective doses. However, the use of antioxidants as a pharmacological approach to treat anxiety or as a co-adjuvant treatment with conventional anxiolytics should be employed with precaution. Indeed, antioxidants at high doses could become toxic by behaving e.g. as prooxidants. In this sense, supplementation of the human diet with high doses of antioxidants, e.g. vitamin C or carotenoids resulted in several adverse effects (reviewed by Bouayed & Bohn, 2010). Therefore, several studies are necessary to determine the safety of antioxidants at high doses, and the duration of the treatment, when the pharmacological approach is envisaged. However, antioxidants from natural foods rich in antioxidants could constitute a preventive therapy against anxiety, owing to their presence at physiological doses. Several animal studies have shown that long-term consumption of honey, apple or olive oil can prevent aging-accompanying anxiety. Nevertheless, the efficiency of antioxidants from fruits and vegetables has been prospectively verified against human diseases related to oxidative stress including coronary heart disease and stroke but has shown to be effective in preventing diseases predominantly when consumed at least at 5 portions per day.

6. References

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3

Pharmacology of 5-HT_2 Modulation of Amygdala & Hypothalamus in Anxiety Disorders

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1. Introduction

"Anxiety disorders" is a blanket term covering several different forms of abnormal and pathological fear and anxiety, and is often comorbid with other mental disorders, particularly clinical depression. These conditions are often related to stressful life experiences, especially when chronic and traumatic. Stress appears to act as a predisposing and precipitating factor in these psychiatric conditions (Strohle and Holsboer, 2003). One particularly, extreme case is post traumatic stress disorder (PTSD), a chronic anxiety disorder developed in the aftermath of traumatic stress exposure and persisting long after the removal of the participating stressors.

Advances in cellular and molecular biology and imaging technology have opened several lines of inquiry into the pathogenesis and pharmacotherapy of the anxiety disorders. Dysregulation of neurotransmitter systems, alteration of signal transduction pathways, and reshaping of brain circuitry are all being explored. The availability of animal models of anxiety disorders developed from the "learned helplessness" stress paradigm, in particular, has been a great aid in elucidation of disease etiology and pathophysiology, as well as in the development of more efficacious pharmacological interventions (Drevets, 2003;Maier and Watkins, 2005;Minor and Hunter, 2002). Among several hypotheses for the pathogenesis of anxiety disorders, dysregulation of the serotonergic system has received particular attention in the field since the evidence from both preclinical and animal model studies is substantial and often complementary. In this chapter, we focus on a subset of the serotonergic system, the 5-HT_2 receptor system, and review both clinical and preclinical evidence regarding the involvement of this receptor in the pathophysiology of anxiety disorders.

2. Neuronal circuitry associated with anxiety disorders

The phenotypic complexity of anxiety disorders indicates that multiple neurotransmitter systems and brain structures are involved in the pathogenesis of such disorders. The

neuronal circuits associated with anxiety disorders appear to involve distributed and interconnected brain structures, including the amygdala, frontal cortex, and hypothalamus. These structures are also principal recipient regions of the ascending serotonergic pathway originating in the dorsal raphé nucleus (DRN), and with this innervation, form the important DRN-corticolimbic pathway in the brain, a critical component of the neuronal network associated with regulation of stress/emotional response (Graeff et al., 1993;Spannuth et al., 2011;Hale et al., 2010;Kawano et al., 1992). Dysregulation of this pathway has long been recognized in the occurrence of stress-related psychiatric syndromes, including depressive disorders and anxiety disorders (Southwick et al., 1999;van Praag, 2004a). Among various serotonin (5-hydroxytryptamine 5-HT) receptor systems, alterations of the postsynaptic 5-HT_2 receptor system in the forebrain may be particularly relevant to the pathophysiology of stress-related psychiatric conditions. There is a general consensus among many PET scan studies that there is decreased forebrain 5-HT_{2A} receptor density in drug-naïve depressed patients (Akin et al., 2004;Malone et al., 2006;Messa et al., 2003;Mintun et al., 2004;Sheline et al., 2004). Several studies also showed that the therapeutic action of antidepressants is associated with an increase and/or normalization in brain 5-HT_{2A} receptor density (Massou et al., 1997;Messa et al., 2003;Sheline et al., 2004;Zanardi et al., 2001). Thus, it is hypothesized that diminished 5-HT_{2A} receptor signaling in the forebrain is associated with the cognitive syndrome observed in PTSD and certain subgroups of depressive illnesses (van Praag, 2004a;van Praag, 2004b).

Animal studies also suggest the involvement of forebrain 5-HT_2 receptor signaling in stress-related psychiatric conditions. For example, activation of 5-HT_{2C} receptors in the amygdala during traumatic stress is necessary for the expression of anxiety-like behaviors after traumatic stress exposure (Christianson et al., 2010). Inescapable stress induces a decrease in 5-HT_{2A} receptor expression in the amygdala, and hippocampus (Dwivedi et al., 2005), and hypothalamus (Dwivedi et al., 2005;Petty et al., 1997;Wu et al., 1999), and the decrease in the number of 5-HT_{2A} receptors in the hypothalamus and hippocampus appears to be specifically associated with behavioral depression after exposure to stress (Dwivedi et al., 2005). In addition, alterations of 5-HT_{2A} receptor signaling in the amygdala have been specifically implicated in the initiation of lasting changes in anxiety-like behavior following predator stress and traumatic stress (Adamec et al., 2004;Jiang et al., 2009). Thus stress-related psychiatric syndromes, including various anxiety disorders, may evolve from altered 5-HT_2 receptor signaling in the forebrain (Graeff et al., 1996;Menard and Treit, 1999).

3. 5-HT_2 receptor expression and its neuronal function in the amygdala

The region of the forebrain involved in anxiety disorders that will be focused on herein is the amygdala, a brain region located deep in the anterior temporal lobe. It is believed that abnormal neural excitability and plasticity in the amygdala is an essential feature of multiple types of anxiety disorders and may be directly linked with the expression of the symptoms associated with stress-related psychiatric conditions. 5-HT_2 receptors appear to be highly expressed in the amygdala (Morilak et al., 1994;Pompeiano et al., 1994;Wright et al., 1995) and thus may serve an important modulatory role in fear and anxiety response. The 5-HT_2 receptor has three subfamilies, including 5-HT_{2A}, 5HT_{2B} and 5-HT_{2C}. Both 5-HT_{2A} and 5-HT_{2C} receptors have been shown to be highly expressed in the amygdala(Xu and Pandey,

2000;Pompeiano et al., 1994;Jiang et al., 2009). The immunofluorescence data from several laboratories show that the 5-HT_{2A} receptor labeling is primarily localized to the soma and dendrites of interneuron-like cells in the basolateral amydala (BLA), and that the majority of the 5-HT_{2A} signal overlapped with the labeling for the interneuron marker parvalbumin, suggesting the 5-HT_{2A} receptor is localized to the interneuron. Interestingly, 5-HT_{2A} receptor immunofluorescence was found to be rarely observed in the pyramidal cells of the BLA, indicating that 5-HT_{2A} receptor expression is restricted to interneurons in the BLA, while the 5-HT_{2C} receptor may be primarily expressed on the pyramidal cells. In addition, the receptors density of various subtypes of 5-HT_2 receptor is dynamically regulated by age, gender, hormones and various experimental conditions associated with anxiety (Chen et al., 1995a;Chen et al., 1995b;Jiang et al., 2009).

The specific expression of 5-HT_{2A} and 5-HT_{2C} receptors in different neuronal components of the amygdala may be related to their specific modulation of neuronal functions in the amygdala and of behavioral responses. Indeed, the observations from several laboratories, particularly our own, support this contention, and activation of 5-HT_{2A} and 5-HT_{2C} receptors induce different neuromodulation in the amygdala and different behavioral responses. Restriction of 5-HT_{2A} receptors to interneurons in the amygdala suggests that 5-HT_{2A} receptors participate in inhibitory modulation of the amygdala circuitry. Indeed, a recent publication has shown that the 5-HT_{2A} receptor is the primary receptor responsible for the serotonerigc facilitation of GABA release in the amygdala (Jiang et al., 2009). Activation of this receptor on amygdala interneurons appears to induce the depolarization of the interneurons and facilitate the GABA release from these neurons (Jiang et al., 2009). Since any mediator facilitating GABAergic synaptic transmission in the BLA should induce an anxiolytic effect, it would be expected that the 5-HT_{2A} receptor is anxiolytic. Activation of this receptor has been observed to induce an anxiolytic effect, although that this action is mediated by the amygdala has not been confirmed (Ripoll et al., 2006;Bourin et al., 2005;Nic Dhonnchadha et al., 2003).

Activation of 5-HT_{2C} receptors in the BLA, in contrast, induce anxiety-like effects in animals (Hackler et al., 2006;Campbell and Merchant, 2003;Antonio Pedro de Mello Cruz et al., 2005,Christianson et al., 2010), suggesting that 5-HT_{2C} receptor activation enhances neuronal excitability in the amygdala. The data from our laboratory suggest that the 5-HT_{2C} receptors may play a modulatory role by promoting NMDA function on pyramidal cells in the amygdala. For example, application of the 5HT_2 receptor agonist 1-(2,5)-dimethoxy-4-iodophen-2-aminopropane (DOI) enhances NMDA receptor-mediated excitatory postsynaptic potentials and calcium influx, and as a consequence, transforms theta-burst stimulated synaptic plasticity from short-term potentiation (STP) to long-term potentiation (LTP) in the BLA (Chen et al., 2003). The facilitating effects of DOI were blocked by the 5-HT_2 receptor antagonist, ketanserin, and by the 5-HT_{2C}-receptor selective antagonist, RS102221 (Chen et al., 2003). Therefore, activation of the 5HT_{2C} receptor may induce anxiety-like effects in animals primarily by enhancing NMDA receptor function in the BLA.

In conclusion, 5-HT_{2A} and 5-HT_{2C} receptors appear to be expressed in the different components of the amygdala neuronal circuitry and have opposite functional roles in modulating the amygdala circuitry and the behavioral responses associated with this circuitry. Pharmacotherapy tailored to modulating the effect of 5-HT_{2A} and 5-HT_{2C} receptors in the BLA may have therapeutic implications in anxiety disorders.

4. Anxiety disorders and dysregulation of 5-HT_2 modulated signaling pathways in the amygdala

Since 5-HT_2 receptors in the amygdala play important neuromodulatory roles in fear and stress responses, dysregulation of 5-HT_2 receptor signaling in the amygdala may result in the abnormal and pathological fear and stress responses manifested in different forms of anxiety disorders. Specifically, any condition promoting 5-HT_{2C} receptor signaling or decreasing 5-HT_{2A} receptor signaling would predispose the amygdala to over-respond to any sensory input, and anxiety status may ensue. Indeed, overexpression of 5-HT_{2C} receptors in forebrain, particularly in the amygdala, has been observed to lead to elevated anxiety in animals (Kimura et al., 2009). Clinical data and preclinical data also suggest that diminished 5-HT_{2A} receptor signaling in the forebrain, including the amygdala, may contribute to pathogenesis of the cognitive syndrome observed in PTSD and certain subgroups of depressive illnesses (van Praag, 2004a;van Praag, 2004b).

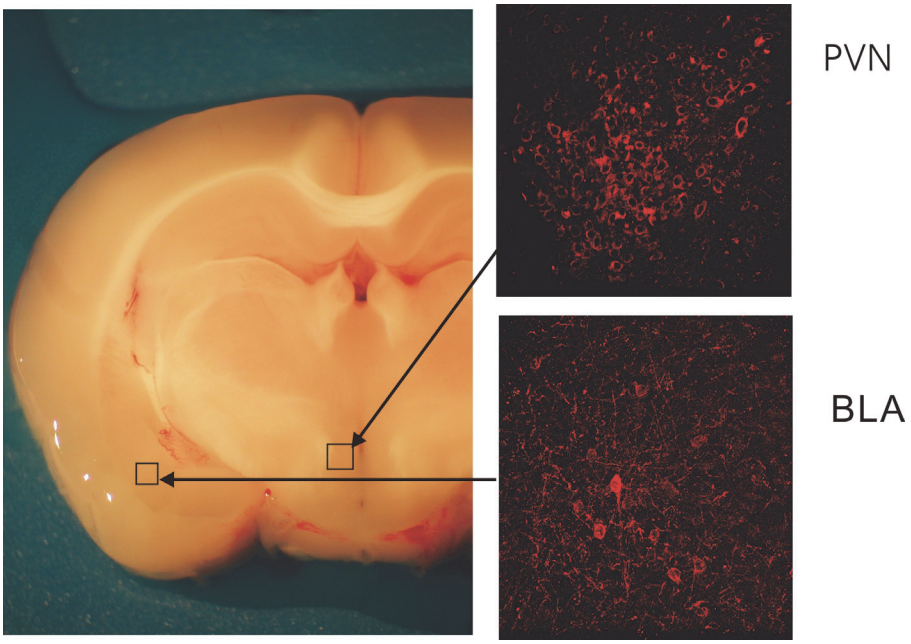

Fig. 1. 5-HT_2 receptors in the BLA and in the paraventricular nucleus (PVN) of the hypothalamus: Immunohistochemistry with specific anti-5HT_2 receptor antibodies reveals 5HT_2 receptors on somata in both the BLA and PVN. In the BLA, 5HT_2 receptors appear on dendrites and axons as well (see text for details.)

More convincing data come from animal studies. Chronic or traumatic stress, a primary etiologic factor for anxiety disorder, particularly PTSD, appears to readily impair central 5-HT_{2A} receptor signaling, including in the amygdala (Abi-Saab et al., 1999;Dwivedi et al., 2005;Jiang et al., 2009;Wu et al., 1999), suggesting that stress induces anxiety in animals by impairing 5-HT_{2A} signaling in the forebrain, particularly in the amygdala. If this is true, then it would be expected that 5-HT_{2A} receptor antagonists, administered during stress, would

prevent the subsequent occurrence of abnormalities remisicent of anxiety disorders in animals since the antagonists would prevent the receptors being downregulated and impaired. Indeed, several laboratories have observed that adminstration of 5-HT_{2A} receptor from antagonists during stress averts several behavorial manisfestations of anxiety status in animals, including exaggerated acoustic startle response and open arm avoidance in the plus maze (Adamec et al., 2004;Jiang et al., 2009). In conclusion, alterations of 5-HT_2 receptor signaling, particularly 5-HT_{2A} receptor signaling in the amygdala, may be a significant contributor in the pathogenesis of anxiety disorders.

Alterations of 5-HT_2 receptor signaling could result from receptor downregulation and degradation, or the disturbance of downstream signal pathways. The 5-HT_2 receptor is a G protein-coupled receptor and activation of the receptor leads to activation of phosphoinositide phospholipase C (PLC) and accumulation of D-myo-inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), each of which then leads to its own signaling cascade, mediating a diverse array of physiological responses (Hall et al., 1999;Schmid and Bohn, 2009). Several studies suggest that distubance of downstream signal pathways of 5-HT_2 receptor may also be involved in the pathophysiology of stress-related psychiatric conditions (Akin et al., 2004). Other potential candidates involved in stress-related psychiatric syndromes are those molecules associated with receptor desensitization and internalization. Like other similar receptors, 5-HT_2 receptors, including abnormal PLC and PKC activity, require the participation of G protein–coupled receptor kinases (GRKs) and β-arrestin in their desensitization and internalization (Schmid et al., 2008;Whalen et al., 2011;Lefkowitz, 1998;Gray et al., 2003; Gray et al., 2001;Bohn and Schmid, 2010), so these molecules could be novel potentially therapeutic targets for anxiety disorders. The most recent finding indeed reveals that β-arrestin-2 is highly expressed in the amygdala and participates in the acquisition and consolidation of fear memories. Manipulation of this molecular signaling pathway thus may be able to regulate the abnormal fear memory associated with certain anxiety disorders (Li et al., 2009).

5. Hypothalamic 5-HT_2 receptors, stress, and energy homeostasis

Another forebrain region critically involved in the pathophysiology of stress-related psychiatric conditions is the hypothalamus. The hypothalamus is a center integrating neuronal and endocrine systems for autonomic functions, including those underlying feeding and behavioral arousal (Jo and Role, 2002a;Gerashchenko and Shiromani, 2004). Different neuronal phenotypes and neurotransmitter systems in the hypothalamus play dynamic roles in maintaining homeostasis and neuroendocrine circadian rhythm in the face of acute and chronic internal and external challenges (Harris et al., 2006a). In addition to multiple neuropeptides, monoamines, and cholinergic and purinergic systems, serotonin plays a critical role in the defensive response to stressful environmental stimuli and energy homeostasis (Jo and Role, 2002a;Jo and Role, 2002b;Pyner, 2009).

The paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin releasing factor (CRF), a key mediator in the stress response, and receives heavy innervation from the serotonergic projection. This nucleus expresses both 5-HT_{2A} and 5-HT_{2C} receptors (Kawano et al., 1992;Li et al., 2003) (Figure 1) and secretion of CRF appears to be regulated by both 5-HT_{2A} and 5-HT_{2C} receptor ligands (Heller and Baraban, 1987;Heisler et al., 2007). These receptors in the PVN are also part of the mechanism mediating feeding and body weight (Leibowitz et al., 1989;Tachibana et al., 2001). Dysregulation of 5-HT_2 receptor systems in the

PVN is thus implicated in anxiety disorders and several affective disorders associated with loss of energy homeostasis.

Indeed, chronic or traumatic stress, a primary etiologic factor for anxiety disorders, readily decreases central 5-HT_{2A} receptor signaling in the hypothalamus, in addition to other forebrain regions (Dwivedi et al., 2005; Petty et al.,1997; Wu et al., 1999), suggesting that stress induces certain physiological abnormalities associated with anxiety disorders, possibly by impairing 5-HT_{2A} signaling in the hypothalamus. One such physiological abnormality is sustained reduced body weight resulting from stress. Sustained body weight loss is a prominent feature observed in animals exposed to different stress paradigms. Weight loss has also been long regarded as a prominent symptom in certain patients with depression and anxiety disorders (Evers and Marin, 2002;Hirschfeld et al., 2005;Hopkinson, 1981). This includes children with anxiety and stress disorder whose growth is stunted (Richards et al., 2006;Yorbik et al., 2004). Since the hypothalamic 5-HT_{2A} receptor is particularly important in stress-related body weight change (Tao et al., 2002;Bah et al., 2010;Rosmond et al., 1998) and mediation of energy homoeostasis (Halder et al., 2007), reduced hypothalamic 5-HT_{2A} receptors may be a determining factor in the occurrence of severe weight loss (Kaye et al., 2005;Kaye et al., 2001;Bailer et al., 2004;Halder et al., 2007). Therefore, stress-induced decrease of 5-HT_{2A} receptors in the hypothalamus may be the underlying mechanism for the sustained body weight loss in stressed animals.

If this is the case, it would be expected that any condition preventing 5-HT_{2A} receptor down regulaton , such as administration of a 5-HT_{2A} antagonist during stress, would be able to avert the subsequent occurrence of sustained body loss in animals. One recent observation appears to support this contention; administration of the 5-HT_{2A} antagonist MDL 11939 during traumatic stress exposure reverses the sustained body weight loss in stressed subjects (Jiang et al., 2009), suggesting that the mechanisms underlying the long-lasting reduction in body weight involve a disturbance of 5-HT_{2A} receptor signaling in certain brain regions, particularly the hypothalamus.

6. Pharmacotherapy for anxiety disorders

Since 5-HT_{2A} receptor and 5-HT_{2C} receptor signaling in the amygdala and hypothalamus may be critically involved in the pathophysiology of anxiety disorders, any agent which is able to specifically modulate 5-HT_{2A} or 5-HT_{2C} receptor signaling in the amygdala and hypothalamus has the potential to treat symptoms associated with various forms of anxiety disorders, including PTSD. Indeed, several clinical studies have shown that the 5-HT_2 receptor antagonist, nefazodone, is effective in improving symptoms of intrusion, avoidance and hyperarousal in a group of Vietnam veterans with chronic-refractory, combat-related PTSD (Neylan et al., 2003;Hertzberg et al., 2002;Garfield et al., 2001;Domon and Andersen, 2000;Zisook et al., 2000;Davis et al., 2000;Mellman et al., 1999;Hidalgo et al., 1999;Davidson et al., 1998). In particular, substantial evidence supports 5-HT_{2A} receptor antagonists for preventing the development of behavioral and physiological changes associated with anxiety disorders , suggesting that these antagonists are promising preventive agents in the fight against stress-associated disorders. Several novel, more selective 5-HT_{2A} antagonists have recently been developed (Bartoszyk et al., 2003) and have been entered into clinical trials for treatments of schizophrenia and insomnia (de Paulis, 2001;Fish et al., 2005). These drugs appear to be well tolerated by all study participants (David et al., 2004) and thus

should also be entered into trials for anxiety disorders, especially PTSD. Among these antagonists, R-96544, a drug metabolized from an orally administrated predrug, R-102444, should be paid particular attention (Ogawa et al., 2005;Ogawa et al., 2004;Ogawa et al., 2002;Tanaka et al., 2008). The pharmacological profile of R-96544 suggests this 5-HT_{2A} receptor antagonist for easy oral administration in the battle field and on site of traumatic events, thus potentially making it an ideal drug for preventing the psychiatric consequences of trauma.

7. Conclusion

Evidence from different disciplines suggests that alterations of 5-HT_2 receptor signaling may be a critical link in the pathogenesis of anxiety disorders. 5-HT_2 receptor signaling in the amygdala and hypothalamus is particularly important in this respect since alterations of receptor signaling in these areas may be directly related to certain symptoms associated with anxiety disorders. Pharmacotherapy tailored to modulating the effect of 5-HT_{2A} and HT_{2C} receptors in the these areas thus represents an important future direction in developing novel, more efficacious pharmacological agents for the symptoms associated with anxiety disorders, including PTSD.

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4

Adenosine Signaling in Anxiety

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1. Introduction

Adenosine is a ubiquitous nucleoside that acts as a neuromodulator in the central nervous system (CNS), controlling neuronal excitability, modulating neurotransmitter release, and regulating ion channel function through four subtypes of G-protein-coupled receptors (GPCRs), A_1, A_{2A}, A_{2B}, and A_3. Adenosine receptor agonists are anxiolytic while adenosine A_1 and A_{2A} receptor antagonists such as caffeine can cause anxiety. Pharmacological and genetic manipulation of A_1 and A_{2A} receptors suggests that each contributes separately to the regulation of anxious states. However, a growing body of evidence argues for a particularly important role of the A_{2A} receptor. Single nucleotide polymorphisms (SNPs) in the A_{2A} receptor gene (ADORA2A) are associated with anxiety in psychiatric disorders and in response to stimulants. Additionally, genetic knockout of the type 1 equilibrative nucleoside transporter (ENT1), which plays an essential role in controlling adenosine levels in the brain, reduces anxiety in rodents, while inhibition of ENT1 may mediate the anxiolytic effects of benzodiazepines and alcohol. In this chapter, we discuss the emerging role of adenosine signaling in anxiety, with special focus on the A_1 and A_{2A} receptors and ENT1. This chapter also includes how caffeine and alcohol regulate anxiety through adenosine signaling.

2. Adenosine in the CNS

Adenosine has several roles in the CNS that are critical to proper brain function. As a nucleoside, adenosine is the precursor to adenine nucleotides in DNA and RNA. It can also be phosphorylated to produce ATP, the main form of cellular metabolic energy. Conversely, it is a product of ATP hydrolysis and as such, represents an indicator of metabolic activity. As a neuromodulator, adenosine can inhibit or excite neurons based upon physiological conditions at the time. Thus, adenosine signaling is best conceptualized as a gating mechanism for signaling by other neurotransmitters, modulating both excitatory and inhibitory neurotransmission. It is in this capacity that adenosine regulates a wide range of behaviors, moods, and emotions (Cunha et al., 2008; Ruby et al., 2010; Asatryan et al., 2011).

Because adenosinergic signaling impacts most neurotransmitter systems in the brain, extracellular adenosine levels must be tightly regulated to support proper neuronal function. Unlike classical neurotransmitters that are synthesized, stored, and released into the synapse in response to electrochemical stimulation, adenosine concentrations are

regulated to a much greater extent by production and transport (Burnstock, 1972, 2006, 2008). This pattern of control allows adenosine levels to change rapidly, which is essential to fine-tune the activity of neighboring neurons. Adenosine reaches extracellular space in two ways: 1) it is produced extracellularly from ATP released by neurons or by astrocytes, and 2) it is released through equilibrative nucleoside transporters (ENTs; **Fig. 1**). Interestingly, astrocytes appear to be significant sources of extracellular adenosine and ATP (Haydon et al., 2009).

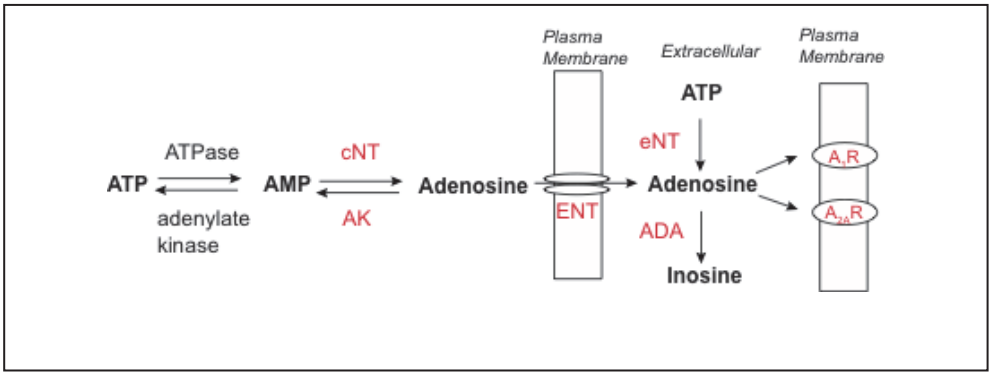

Fig. 1. Schematic showing adenosine production in the central nervous system. Abbreviations: cNT: cytosolic endo-nucleotidase, AK: adenosine kinase, ENT, equilibrative nucleoside transporters, eNT: exo-nucleotidase, ADA: adenosine deaminase, A_1R and $A_{2A}R$: adeonsine A_1 and A_{2A} receptors.

Adenosine controls neurotransmitter release, modulates neuronal excitability and regulates ion channel function through four subtypes of GPCRs, A_1, A_{2A}, A_{2B}, and A_3, all with distinct affinity for adenosine. Whether adenosine exerts a dampening or potentiating effect on neurotrasmission is determined by the expression pattern of adenosine receptors and the levels of adenosine in the brain (Fredholm et al., 1999; Fredholm et al., 2005b; Fredholm et al., 2005a; Fredholm, 2010). A_1 and A_{2A} receptors have 10-100 nM binding affinities, whereas A_{2B} and A_3 receptors have 1-5 mM binding affinities. Since normal CNS adenosine levels are 25-250 nM, A_1 and A_{2A} receptors are the main subtypes involved in the regulation of anxiety and other psychiatric disorders. Adenosine A_1 receptors are expressed ubiquitously in the CNS, have high affinity for adenosine, and mediate tonic inhibition of neuronal activity. Activation of presynaptic A_1 receptors inhibits the release of excitatory and inhibitory neurotransmitters by reducing intracellular cAMP and PKA activation, while postsynaptic A_1 receptors regulate potassium channels to reduce both excitability (the probability of firing) as well as action potential duration (Ebersolt et al., 1983; Fredholm, 1985; Linden, 1991; Heurteaux et al., 1995). Adenosine A_{2A} receptors are primarily expressed in the caudate-putamen and nucleus accumbens. In contrast to A_1 receptors, A_{2A} receptors are positively linked to adenylate cyclase, increasing levels of cAMP and exerting excitatory influences on neurons. A_{2A} receptors are also known to associate physically with other neurotransmitter receptors, including the dopamine D2 and glutamate mGluR5 receptors. Such receptor-receptor interactions appear to be essential for striatal function, and evidence suggests that they may be impaired in a number of psychiatric diseases (Ferre et al., 2010).

3. Adenosine receptors and transporters in anxiety

Adenosine and adenosine receptor agonists are anxiolytic as assessed by a number of ethological tests in rodent models, such as the elevated zero maze and elevated plus maze (Kulkarni et al., 2007), the Vogel conflict test (Okuyama et al., 1999), and the light-dark (LD) box (Florio et al., 1998). Conversely, adenosine receptor antagonism is responsible for the anxiogenic responses elicited by moderate to high doses of caffeine, theophylline (Imaizumi et al., 1994; Kulkarni et al., 2007; Pechlivanova et al., 2010), or other adenosine receptor antagonists (Imaizumi et al., 1994; Florio et al., 1998; Koetter et al., 2009; Zhao et al., 2009) in rodents and humans (Lara, 2010). It has also been suggested that the anxiolytic effect of the adenine derivative BWA78U in the LD box involves adenosine receptor activation (Willard et al., 1990), and that adenosine is the active principle in the Longan Arillus extract (Okuyama et al., 1999), a traditional Asian remedy for mild anxiety. Moreover, adenosine may mediate, in part, the anxiolytic activity of benzodiazepine receptor ligands (Snell and Snell, 1984; Stone, 1999). Genetic evidence supports roles for the A_1 and A_{2A} receptors, as well as the transporter ENT1 in the regulation of anxious states.

3.1 The A2A receptor

Of the many studies implicating adenosine signaling in emotional behavior, the strongest evidence favors a central role for the A_{2A} receptor subtype in anxiety-related disorders under many different conditions (Shen and Chen, 2009). The A_{2A} receptor is enriched in the caudate-putamen and nucleus accumbens, where it interacts physically with dopamine D2 receptors to regulate reward, habitual behavior, and locomotor activity (Cunha et al., 2008). As the A_{2A} receptor is expressed in both neurons and astrocytes, it has also been implicated in controlling glial function and brain metabolic adaptation, processes that are dysregulated in several psychiatric disorders (Lee et al., 2007; Rajkowska and Miguel-Hidalgo, 2007).

Persuasive preclinical evidence for the involvement of the A_{2A} receptor in anxiety comes from several studies based on its genetic deletion combined with pharmacological manipulation. Indeed, A_{2A} receptor null mice are considered a valuable model to study anxiety disorders and develop new therapies (Deckert, 1998). These mice display reduced exploratory activity, heightened anxiety and aggression, hypoalgesia, increased blood pressure and heart rate, and aberrant locomotor responses to caffeine (Ledent et al., 1997), including caffeine-induced depression (rather than enhancement) of exploration (Ledent et al., 1997), and the absence of an anxiogenic response to acute or chronic high-dose caffeine in the elevated plus maze (El Yacoubi et al., 2000). This evidence is in line with a pharmacological study showing that the adenosine A_2 receptor agonist CGS21680 reduced the anxiogenic effect of theophylline in the LD box (Imaizumi et al., 1994). Surprisingly, however, A_{2A} receptor overexpression did not alter anxiety-like responses in the elevated plus maze (Gimenez-Llort et al., 2007).

Pharmacological and genetic inhibition of the A_{2A} receptor has also revealed its role in the regulation of anxiety in disease states, by other neuromodulators, or by drugs of abuse. One study showed increased content of a-MSH, a pro-opiomelanocortin (POMC)-derived peptide known to influence anxiety, aggressive behavior, and motor activity, in the amygdala and cortex of A_{2A} receptor knockouts (Jegou et al., 2003). The mice also had augmented levels of POMC mRNA and ACTH in the anterior pituitary, indicating hyperactivity of the hypothalamic-pituitary-adrenal axis that mediates responses to stress (Jegou et al., 2003). The A_{2A} receptor has also been implicated in the anxiolytic activity of

prostaglandin D2, as A_{2A} receptor inhibition by SCH58261 prevented the increase in open-arm time produced by prostaglandin D2 in the elevated plus maze. In addition, A_{2A} receptor blockade prior to quinolinic acid-induced striatal excitotoxic lesions (a rat model of Huntington's disease), prevented the usual increase in anxiety-like behavior for 6 months (Scattoni et al., 2007). Moreover, the A_{2A} receptor may be involved in anxiety during morphine withdrawal, as A_{2A} receptor null mice display more severe naloxone-precipitated withdrawal symptoms (Berrendero et al., 2003). Finally, these mice are more sensitive to the anxiolytic properties of alcohol (Houchi et al., 2008). These studies and other research implicating adenosine signaling in the effects of alcohol are discussed later in the chapter.

Disorders	SNPs	References
Panic Disorder	1083C>T	Hamilton et al., 2004
	1976C>T	
	rs5751876	Hohoff et al., 2010
Agoraphobia	1083C>T	Hamilton et al., 2004
	rs5751876	Hohoff et al., 2010
Blood-Injury Phobia	1976C>T	Hohoff et al., 2009
Autism Spectrum Disorder	rs2236624CC	Freitag et al., 2010
	rs3761422	
	rs5751876	
	rs35320474	
Caffeine-Induced Anxiety	1976C>T	Alsene et al., 2003
	2592C>T	
	rs5751876	Childs et al., 2008; Rogers et al., 2010
	rs2298383	Childs et al., 2008
	rs4822492	
Amphetamine-Induced Anxiety	1976C>T	Hohoff et al., 2005
	2592C>T	
Anxiety-Related Personality	rs5751862	Hohoff et al., 2010
	rs2298383	
	rs3761422	

Table 1. Single-nucleotide polymorphisms (SNPs) in ADORA2A, the gene that encodes the adenosine A2A receptor, are involved in a number of anxiety-related psychiatric disorders.

Perhaps most compelling is the large body of clinical evidence demonstrating that a variety of single nucleotide polymorphisms (SNPs) in the A_{2A} receptor gene, ADORA2A, is associated with anxiety in several psychiatric disorders and under drug-challenge conditions (**Table 1**). Panic disorder has been associated in different studies with several of ADORA2A SNPs including 1083C>T (Hamilton et al., 2004), rs5751876 (Hohoff et al., 2010), and 1976C>T (Hamilton et al., 2004). Association of the 1976C>T SNP with panic disorder was not replicated in a Chinese population (Lam et al., 2005), although the sample numbers were relatively low (>300 total individuals). It is noteworthy that the 1976C>T variant was also associated with self-reported anxiety after a moderate dose of orally-administered caffeine (150 mg; Alsene et al., 2003), amphetamine (10-20 mg; Hohoff et al., 2005), and sympathetic nervous system activation in individuals with blood injury phobia (Hohoff et

al., 2009). In addition to panic disorder and agoraphobia, the rs5751876 genetic variant of ADORA2A was also associated with autism spectrum disorders (ASD) (Freitag et al., 2010) and caffeine-induced anxiety (Childs et al., 2008). Interestingly, a recent study found that people with the rs5751876TT genotype, although more susceptible to caffeine-induced anxiety, habitually drank more coffee, concluding that tolerance to the anxiogenic effect of caffeine occurs regardless of susceptibility (Rogers et al., 2010). Two other variants of the A_{2A} receptor gene, rs2298383 and rs3761422, were found to be associated with multiple anxiety-related personality scores (Hohoff et al., 2010), with the former also involved in caffeine-induced anxiety (Childs et al., 2008), and the latter influencing phenotypic variability in ASD symptoms (Freitag et al., 2010). Other variants, rs2236624CC and rs35320474 (Freitag et al., 2010), associated more specifically with ASD, while rs4822492 was related to caffeine-induced anxiety (Childs et al., 2008), and rs5751862 was related more broadly to anxious personality (Hohoff et al., 2010). Although the effect of these SNPs on the expression or function of the A_{2A} receptor is not yet known, it is clear that this receptor is crucial to the regulation of anxiety.

3.2 The A1 receptor

In contrast to the A_{2A} receptor, a role for the A_1 receptor has been more difficult to establish. Genetic knockout of the A_1 receptor appears to argue for this receptor mediating the anxiolytic activity of adenosine, while approaches relying solely on pharmacological methods have been less clear. Moreover, no studies to date have implicated genetic variants of the A_1 receptor in anxiety disorders in humans.

Pharmacological regulation of the A_1 receptor has borne mixed results, with some studies demonstrating anxiolytic properties of its activation, others showing anxiolysis produced by its inhibition, and still others showing no effect of A_1 ligands on anxiety measures at all. Moreover, the differences in these studies are difficult to reconcile on the basis of drug selectivity or dose. For example, one study showed that A_1 receptor agonist CCPA was anxiolytic in the elevated plus maze and LD box, while A_1 receptor antagonists CPT and IBMX were anxiogenic in these tests (Florio et al., 1998). Another study showed that the anxiogenic effect of A_1-selective antagonist DPCPX was unaffected by A_1 agonist CPA. Yet another study showed the both CPA and CPX (A_1 antagonist) decreased the anxiolytic activity of ifenprodil (a glutamate NMDA antagonist; Fraser et al., 1996). Likewise, antagonism of A_1 receptors has been implicated in the actions of magnolia and ziziphus extracts, traditional Eastern treatment of mild anxiety and nervousness (Koetter et al., 2009).

Where pharmacology has failed to illustrate a consistent role for the A_1 receptor in anxiety-like behavior, genetic methods have yielded a much clearer picture. Deletion of the A_1 receptor gene in mice results in increased measures of anxiety in several different behavioral assays, including decreased exploration in the open-field and hole board, reduced open arm entries and time in the elevated plus maze, less time in the light portion of the LD box (Gimenez-Llort et al., 2002), and increased wall-hugging in the water maze (Lang et al., 2003). A_1 receptor knockouts also show a reduction in adenosine-mediated inhibition of glutamate neurotransmission and abolishment of theophylline-induced enhancement of glutamatergic signaling (Johansson et al., 2001). Other changes in these mice include reduced activity during some phases of the LD cycle, and reduced muscle strength and survival (Gimenez-Llort et al., 2002), indicating a possible role for the A_1 receptor in aging-related deficits, despite the normal spacial performance by the mice (Lang et al., 2003). Additionally, mice lacking the preproenkephalin gene, that show decreased locomotor activity, hyperalgesia, increased

anxiety and aggression, have enhanced central A_1 receptor-specific DPCPX binding, presumably reflecting an attempt to counteract or balance the loss of endogenous opioids (Bailey et al., 2004). These lines of evidence support the notion that the A_1 receptor does indeed regulate anxiety, and that its activation produces an anxiolytic behavioral response.
There is also preclinical evidence that the A_1 receptor may be involved in anxiety-like behavior in stressed animals and rodent models of hyperthyroidism. Rats subjected to a 3-day stress procedure showed a 15% increase in A_1 receptor binding in hypothalamic membrane preparations, as well as higher plasma corticosterone (Anderson et al., 1987). Thyroid hormones affect the development, function, and expression of A_1 receptors and regulate the transport of adenosine in the brain (Fideu et al., 1994), a hallmark of hyperthyroidism is anxiety. Hyperthyroidism induction was shown to have lasting effects on nucleotide hydrolysis (and thus, the availability of adenosine) in the rat brain, with young rats showing decreases of 14-52% in the hippocampus and cortex, while older rats had increased AMP hydrolysis in the cortex, but lasting decreases in the hippocampus (Bruno et al., 2003). Because CPA reduced anxiety-like behavior in hyperthyroid rats (Bruno et al., 2006), reduced A_1 receptor activation may underlie anxiety in hyperthyroidism.

3.3 ENT1

As discussed above, nucleoside transport is one of the most important determinants of adenosine levels in the CNS (Dunwiddie, 1985; Burnstock, 2008). Of the several equilibrative and concentrative transporters expressed in the brain, ENT1 appears to be central to the regulation of anxious states. Some of the first evidence in support of this idea came from a study demonstrating that pharmacological inhibition of adenosine uptake by papaverine was anxiolytic in the elevated plus maze test (Zangrossi et al., 1992). This study also suggested that inhibition of adenosine uptake may be a mechanism of the anxiolytic drug carbamazepine, as its actions were inhibited by adenosine receptor antagonist aminophylline (Zangrossi et al., 1992). It is noteworthy that multiple studies have also pointed to inhibition of ENT1 as a mechanism for benzodiazepine-induced anxiolytic activity (discussed later). Recently, we showed that mice lacking ENT1 display decreased baseline anxiety levels in the open-field, elevated plus maze, and LD box tests (Chen et al., 2007). Moreover, decreased anxiety in the open-field and elevated plus maze was replicated in C57BL/6J mice after microinjection of the ENT1 inhibitor NBTI (also called NBMPR) into the amygdala (Chen et al., 2007). NBTI binding was also upregulated in the brain following deletion of the preproenkephalin gene in mice, a manipulation which increases anxiety and aggression (Bailey et al., 2004). Increased ENT1 levels were accompanied by increases in A_1 receptor levels in this study, with these changes presumably an adaptive response to the loss of opioid peptides (Bailey et al., 2004). One clinical study undertaken to test the effect of dipyridamole on anxiety demonstrated no measurable improvement in patients with generalized anxiety disorder or panic disorder (Stein et al., 1993), although the sample size was extremely low (under 20 patients), and dipyridamole is not a very specific drug, acting as an inhibitor of both adenosine uptake and adenosine deaminase. The role of ENT1 and other nucleoside transporters in regulating anxiety clearly warrants future investigation.

4. Adenosine-GABA interactions in anxiety

Another well-known neurotransmitter system involved in the regulation of anxiety is the GABAergic system (Baldwin and File, 1989; Crestani et al., 1999; Kash et al., 1999; Löw et al.,

2000; Hodge et al., 2002). The relationship between GABAergic and adenosinergic signaling is interesting, because there is considerable functional overlap between their actions, such as the reduction of neuronal excitability. However, in contrast to the globally inhibitory actions of GABA upon neurons in the adult brain, adenosine can act as a permissive factor for signaling by other neurotranmitters, excitatory and inhibitory alike. Hence, the actions of adenosine on GABAergic signaling are similar to its actions on glutamatergic signaling, and whether adenosine dampens or potentiates GABA effects depends upon the expression site of adenosine receptors and their differential activation by ever-fluctuating adenosine concentrations. Given the implication of both GABA and adenosine signaling in decreasing anxiety, it is surprising that little research has focused on how these systems interact in anxiety disorders. The benzodiazepine anxiolytics, which potentiate signaling through the $GABA_A$ receptor, also interact with the adenosinergic system at several levels. These interactions represent the main focus of this section.

4.1 Benzodiazepines and adenosine signaling

In addition to their potentiation of $GABA_A$ receptor-mediated signaling, benzodiazepines (BZDs) are known to inhibit adenosine uptake, and several authors have suggested that this action may underlie, at least in part, the anxiolytic effect of these agents (Bruns et al., 1983; Phillis, 1984; Phillis and O'Regan, 1988). Indeed, diazepam and adenosine share a similar physico-chemical structure (Bruns et al., 1983). Inhibition of adenosine uptake by BZDs, including diazepam, lorazepam, and flurazepam, has been demonstrated in different experimental preparations, including rat brain synaptosomes (Phillis et al., 1980) and guinea pig ventricle (Barker and Clanachan, 1982). A study showing that BZDs prevent NBTI binding revealed ENT1 as a specific target of these drugs (Hammond et al., 1981). This is consistent with the reduced anxiety-like behavior in ENT1 knockout mice (Chen et al., 2007) and the anxiolytic action of adenosine uptake inhibitor papaverine (Zangrossi et al., 1992).

Given their structural similarities with adenosine, it is not surprising that BZDs have also been shown to interact with adenosine receptors in some cases. Furthermore, modifications in adenosine signaling have been linked to BZD withdrawal responses in animal models. Both the A_1 and A_2 subtypes of adenosine receptors appear to be affected by BZD treatment, but most evidence points to the A_1 receptor as playing a larger role in BZD action. BZDs did not displace chloroadenosine from A_1 receptors, indicating that direct action on these receptors is not likely responsible for BZD-mediated anxiolytic activity (Williams et al., 1981). Chronic treatment with mixed A_1/A_{2A} receptor antagonists, caffeine or theophylline, reduced GABA potentiated flunitrazepam binding to the BZD site on the $GABA_A$ receptor (Roca et al., 1988). Since this action was blocked by chloroadenosine (Roca et al., 1988), it appears that A_1 receptor activation is required for the full potentiation of $GABA_A$ receptor signaling by BZDs. Studies on BZD withdrawal also indicate that A_1 receptor-mediated responses are integral to the effects of these anxiolytics. For example, administration of either caffeine or selective A_1 receptor antagonist DPCPX intensified BZD withdrawal in mice (Listos et al., 2006). Additionally, A_1 receptor agonist CPA was more efficacious in attenuating BZD withdrawal signs in mice than A_{2A} receptor agonist CGS (Listos et al., 2005). Both studies support at least a minor role of A_{2A} receptors in the actions of BZDs, which is in agreement with an older study showing displacement of adenosine from A_2 receptors by BZDs in neuroblastoma x glioma hybrid cells (Snell and Snell, 1984). In this same study, diazepam facilitated A_2-mediated cAMP production, but had no effect on this

measure in the absence of adenosine (Snell and Snell, 1984). While the exact contribution of A_1 and A_2 receptors in the effects of BZDs is not known, it appears a role exists for endogenous adenosine in the anxiolytic properties of this class of drug. This is consistent with the elevated availability of adenosine that results from ENT1 blockade by BZDs (discussed previously). Finally, this evidence indicates that BZDs might lose anxiolytic efficacy in habitual caffeine or theophylline consumers, and that people withdrawing from BZDs should avoid coffee, tea, and cola.

4.2 Other adenosine-GABA interactions

Adenosine signaling has also been implicated in the effects of other types of anxiolytic compounds that are known to potentiate $GABA_A$ receptor-mediated neuronal inhibition. Carbamazepine, an anxiolytic and anticonvulsant drug, reduces neuronal excitability in several ways, including stabilization of the inactivated state of sodium channels and $GABA_A$ receptor activation. Evidence for the involvement of adenosine in carbamazepine-mediated anxiolytic activity comes from a study showing that nonselective adenosine receptor antagonist aminophylline blocked the increase in open-arm time in the elevated plus maze in mice (Zangrossi et al., 1992). It is conceivable that adenosine acts as a permissive factor for $GABA_A$ activation, similar to the manner in which adenosine appears to partially mediate BZD action. Another study citing synergism between adenosine and $GABA_A$ signaling in reducing anxiety demonstrated that the anxiolytic activity of prostaglandin D2 in the elevated plus maze test in mice was blocked by both A_{2A} receptor antagonist SCH58261 and $GABA_A$ receptor antagonist bicuculline (Zhao et al., 2009). The synergism between adenosine and GABA in regulating anxiety remains an interesting and potentially important future prospect in the field of anxiety disorders.

5. Regulation of adenosine signaling by caffeine and alcohol

Caffeine and alcohol are the two most commonly used psychoactive substances in the world. Research has revealed that adenosine signaling is central to the anxiety regulating effects of these drugs. While people can benefit from many of the effects of adenosine receptor antagonism by caffeine, such as increased alertness, improved attention or focus, and even amelioration of depressive symptoms (Lara, 2010), sensitivity to caffeine-induced anxiogenesis may preclude certain individuals from enjoying coffee, tea, cola, or chocolate. On the other hand, moderate doses of alcohol are anxiolytic, and sensitivity to this effect may lead a person to abuse alcohol. There is a large body of information indicating that several of the CNS depressant effects of alcohol, including anxiolytic activity, are mediated by adenosine. In this section, the regulation of adenosine signaling by these substances will be discussed, with emphasis on how such action influences anxious behavior.

5.1 Caffeine

Much of what is currently known about adenosine signaling in general is based on studies using caffeine. Caffeine exerts its stimulant effects on the CNS by inhibiting adenosine A_1 and A_{2A} receptors, as does the related compound theophylline, and other synthetic methylxanthine derivatives. Caffeine can also inhibit phosphodiesterases and mobilize intracellular calcium, but the doses required for such actions are enormous, and not physiologically relevant (Nehlig et al., 1992). High (but physiological) doses of caffeine

cause anxiety in most people, low doses go essentially unnoticed, but significant individual differences exist in sensitivity to moderate doses of caffeine. Such individual differences in response to caffeine have been linked specifically to genetic polymorphisms in the A_{2A} receptor gene (ADORA2A), which may affect the expression or function of the receptor. Self-reported anxiety after moderate caffeine intake (150 mg, oral) was associated with ADORA2A variants 1976C>T and 2592C>T (Alsene et al., 2003). Individuals with the ADORA2A SNP rs5751876TT also had greater susceptibility to caffeine-induced anxiogenesis (Rogers et al., 2010). A puzzling observation in this study was that these genetically susceptible people tended to drink more coffee habitually, and that moderate to high habitual consumers of caffeine experienced less caffeine-induced anxiety, irrespective of genotype (Rogers et al., 2010). Thus, it appears that the history of caffeine exposure itself is a better predictor of whether or not someone will feel anxious when consuming caffeine. However, several SNPs in the ADORA2A gene, including those influencing responses to caffeine, are also associated with panic disorder, agoraphobia, autism, and amphetamine-induced anxiety (discussed previously), consistent with an older study showing that the majority of patients with agorophobia and panic disorder find caffeine to be anxiogenic (Charney et al., 1985). The results of these genome association studies underscore the importance of the A_{2A} receptor in several manifestations of anxiety.

Preclinical studies on the effects of caffeine largely support the clinical observations, including the central role of the A_{2A} receptor in caffeine-induced anxiogenesis. Caffeine, theophylline, and DPCPX were anxiogenic in the LD box test, with these effects reduced by A_2 agonist CGS21680, but not by A_1 agonist CPA (Imaizumi et al., 1994). Moreover, high-dose caffeine treatment, both acutely and chronically administered, failed to induce anxiety in A_{2A} receptor knockout mice in the elevated plus maze test (El Yacoubi et al., 2000). Prenatal caffeine exposure in Sprague-Dawley rats reduced anxiety in the elevated plus maze and the LD box, and enhanced responses to A_{2A} receptor agonist CGS21680 (Pan and Chen, 2007). Pretreatment with caffeine or theophylline reversed the anxiolytic effect of adenosine in the elevated plus maze and elevated zero maze (Kulkarni et al., 2007). Interestingly, despite being anxiogenic in Wistar rats in the elevated plus maze, caffeine actually increased open arm time after the rats underwent a chronic, unpredictable stress procedure (Pechlivanova et al., 2010), a method to model depression in rodents. This reduction in anxiety-like behavior by caffeine in the context of depression may be related to other evidence suggesting that moderate caffeine intake (< 6 cups/day) was associated with less depression and a lower risk of suicide (Lara, 2010). This suggests that different mechanisms may underlie the pathogenesis of anxiety and depression, despite their co-occurrence in many psychiatric diseases, and that both may involve changes in adenosine signaling.

5.2 Alcohol

Adenosine is known to contribute to many of the intoxicating effects of alcohol, such as its ataxic and sedative properties (Ruby et al., 2010; Asatryan et al., 2011). In general, sensitivity to the aversive effects of ethanol is inversely correlated with alcohol consumption. Indeed, the importance of adenosine signaling in the subjective effects of alcohol has been illustrated recently by the tragic deaths of several college students who were drinking the caffeinated alcoholic beverage,Four Loko, which has subsequently been taken off the market by the FDA. The incredibly high blood alcohol levels achieved by drinkers of Four Loko (0.4%)

reflected caffeine's ability to decrease sensitivity to the stumbling and tiredness associated with drinking large quantities of alcohol. Importantly, adenosine also appears to mediate some of the reinforcing effects of alcohol, including its well-known ability to reduce feelings of anxiety. Increased sensitivity to rewarding or reinforcing effects of ethanol is associated with greater drinking.

Ethanol reinforcement is in part mediated by A_{2A} receptor activation and associated intracellular signaling cascades in the nucleus accumbens (Adams et al., 2008), but the exact contribution of A_{2A} receptor-mediated signaling to drinking behavior remains unclear. A_{2A} receptor knockout mice show hyposensitivity to the intoxicating effects of ethanol and self-administer more alcohol than do wild-types (Naassila et al., 2002). As discussed previously, these mice also display increased basal anxiety, a potential contributing factor to their drinking behavior. Despite the counterintuitive observation that A_{2A} null mice showed reduced conditioned place preference for ethanol, they demonstrated increased sensitivity to the anxiolytic and locomotor stimulating (ie. pleasant) effects of alcohol, which may explain their greater ethanol self-administration (Houchi et al., 2008). Furthermore, the A_{2A} receptor agonist CGS21680 reduced alcohol consumption and preference in C57BL/6J mice (Houchi et al., 2008). Another study showed that A_{2A} receptor antagonist DMPX dose-dependently decreased lever-pressing for ethanol in an operant chamber, but had no effect on anxiety measures in the elevated plus maze or Vogel conflict assessments (Thorsell et al., 2007). However, yet another study showed that A_{2A} receptor antagonist ZM241385 had no effect on the anxiolytic activity of ethanol, suggesting instead that the A_1 receptor mediates this effect (Prediger et al., 2004). The contradictory results of these studies may reflect differences in specificity of the adenosinergic drugs administered, or general differences in approach between genetic and pharmacological studies. Alternatively, it may reflect a missing factor that affected the balance of adenosine signaling in opposite ways. Whether or not polymorphisms in ADORA2A are associated with alcohol intake patterns in humans is not yet known, so this is an interesting future prospect.

The adenosine transporter ENT1 appears to be involved in many aspects of alcohol-related behaviors (Choi et al., 2004; Chen et al., 2010; Nam et al., 2010; Nam et al., 2011) and anxiety (Chen et al., 2007). Moreover, a recent study showed that a polymorphism in the gene encoding ENT1 is associated with alcoholism and depression in women (Gass et al., 2010) and alcoholics with a history of withdrawal seizures (Kim et al., 2011). Acute ethanol inhibits ENT1, while chronic alcohol treatment leads to decreased ENT1 expression (Short et al., 2006; Sharma et al., 2010). This action of ethanol appears to be related to its ability to produce anxiolysis, as ENT1 null mice display decreased anxiety-like behavior in the open-field, elevated plus maze, and LD box (Chen et al., 2007). Microinjection of ENT1-specific inhibitor NBTI into the amygdala of C57BL/6J mice similarly reduced anxiety in the open-field and elevated plus maze tests (Chen et al., 2007). Interestingly, both manipulations resulted in increased alcohol consumption and preference, indicating that decreasing anxiety (negative reinforcement) does not appear to play a large role in the motivation for alcohol in this model. Since ENT1 null mice also show reduced conditioned place aversion for ethanol (Chen et al., 2010), their high alcohol drinking may be in part related to a lack of "healthy" amounts of anxiety and aversion that would normally prevent them from consuming large amounts of ethanol (ie. a greater degree of impulsivity). The data on ENT1 null mice is consistent with the evidence presented earlier in the chapter suggesting that ENT1 inhibition may be a mechanism by which benzodiazepines exert their anxiolytic

effects. As both high anxiety and low anxiety are associated with increased alcohol drinking behavior in studies of adenosine signaling, perhaps this apparent paradox highlights the importance of appropriate degrees of anxiety (and well-balanced adenosine signaling) in preventing excessive alcohol intake.

Abberrant adenosine signaling is also likely related to anxiety responses during ethanol withdrawal. Adenosine agonist R-PIA decreased open arm time in the elevated plus maze, while antagonist CPT produced partial recovery from ethanol withdrawal-induced anxiety (Gatch et al., 1999). CPT itself was anxiolytic in the LD box in rats, but did not reduce their ethanol consumption or preference (Gatch et al., 1999). A more recent study showed that adenosine and A_1 receptor agonist CCPA, at doses that were not normally anxiolytic, reduced peak-time ethanol hangover-induced anxiety in the elevated plus maze (Prediger et al., 2006). The effect of CCPA was reversed by pretreatment with A_1 receptor antagonist DPCPX, while A_{2A} receptor agonist DPMA had no effect (Prediger et al., 2006). Differences between these studies may reflect the specificity of the adenosine ligands used, or the time and intensity of ethanol exposure.

6. Conclusion

Adenosine is a ubiquitous CNS neuromodulator that regulates the signaling of major neurotransmitter systems involved in mood and emotion. Adenosine and adenosine receptor agonists are anxiolytic, while antagonists such as caffeine, are anxiogenic at high doses in most people, or at moderate doses in susceptible individuals. The availability of adenosine is largely regulated by nucleoside transporters such as ENT1, whose inhibition by benzodiazepines and alcohol may underlie their anxiolytic actions. Research also implies that adenosine-mediated signaling potentiates activation of the $GABA_A$ receptor, another target of anxiolytic drugs. Two types of adenosine receptors, the A_1 and A_{2A} subtypes, appear to contribute differentially to the regulation of anxious states. Both preclinical evidence and genome association studies strongly suggest that the A_{2A} receptor plays a central role in anxiety-related disorders, including panic disorder with agoraphobia, autism spectrum disorder, and anxiogenic responses to stimulants. Multiple lines of evidence support that deviation from a relatively narrow range of adenosinergic signaling balance may contribute to the development of many psychiatric conditions linked with anxiety, including depression and alcoholism.

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5

PTSD and Current Translational Research

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1. Introduction

Posttraumatic stress disorder (PTSD) is a chronic and disabling anxiety disorder that occurs after a traumatic event. In this chapter, we will briefly discuss current research in concept, diagnosis and treatment strategies of PTSD. In addition, we will introduce the concept of biomarkers for PTSD. In each section, we will focus on the discussion of current translational research in PTSD, including clinical and molecular studies in PTSD. Specifically, we will discuss the strategies of translational studies, from bench to bedside and from bedside to bench, two research approaches in the development or identification of PTSD biomarker, a novel approach to diagnosis and treatment. Recent data from the national co-morbidity survey indicates PTSD prevalence rates are 5% and 10% respectively among American men and women. The prevalence of PTSD in the military population is much higher from 10% to 30%.

2. Concept of PTSD

PTSD is a type of anxiety disorders. Its onset may occur soon after a major trauma, or be delayed by more than 6 months after the event. When PTSD occurs, it usually gets better after 3 months. However, some people will suffer chronically from PTSD, lasting for many years. PTSD can occur at any age and can follow a natural disaster such as a flood or fire, or events such as war, a prison stay, assault, domestic abuse, and/or rape. The terrorist attacks of September 11, 2001, for example, caused PTSD in people who were involved, witnessed the disaster, or lost relatives and friends. PTSD can also occur in military service members during war, such as the Iraq war. These traumatic events can produce stress in all involved, but less than 30% of those involved develop PTSD. PTSD patients exhibit depressive symptoms, abnormality of the circulating levels of the stress hormones and neurotransmitter activity, and alteration of gene expression. PTSD symptoms have been around for long time. The significance of PTSD came to public attention only recently.

3. Historical changes in the concept of PTSD in diagnosis

Throughout our history, PTSD has been called a number of other different names. It was called soldier's heart for soldiers who developed the symptoms of PTSD after the Civil War and combat fatigue or shell shock for soldiers after World War I. During the World War II, it

was called battle fatigue or gross stress. PTSD was recognized as one of mental disorders by the American Psychiatric Association (APA) officially and was added to the Diagnostic Manual of Mental Disorders (DSM), in 1980s. The DSM-III diagnostic criteria for PTSD were revised in DSM-III-R (1987) and DSM-IV (1994). A very similar syndrome is classified in ICD-10.

It is the Vietnam War that brought significant public attention to this emotional disorder. In 1980, PTSD was recognized as a disorder by the American Psychiatric Association (APA) and was added to the Diagnostic Manual of Mental Disorders (DSM). In fact, before PTSD was recognized as an emotional disorder, it was considered to be nothing more than cowardice or personal weakness[1].

PTSD also has one or more comorbidities. These common co-morbid diagnoses include major affective disorders (MDD), bipolar disorder, dysthymia, alcohol or substance abuse disorders, anxiety disorders and personality disorders. The mechanisms underlying the high rate of co-morbidity seen with PTSD are still unknown. There are no exclusionary criteria in DSM-III-R. Diagnostic criteria for PTSD include a history of exposure to a "traumatic event" and symptoms from each of three symptom clusters: intrusive recollections, avoidant/numbing and hyper arousal symptoms. Duration of symptoms is considered as a fifth criterion. The following are the common symptoms - a three-factor PTSD structure in the DSM system.

4. Current studies of PTSD symptom models and diagnosis

Although the PTSD symptoms of three-factor (re-experience, avoidance/numb, and hyper-arousal) has been considered as the diagnostic categories in the DSM system, several studies found three factors to be insufficient[2] and proposed several alternative models. In 1998, King et al[3] proposed a model of four-factor, which comprises re-experience, avoidance, emotional numbing, and hyper-arousal by separating the Avoidance/Numbing factor into two factors: Avoidance and Emotional Numbing. Their studies are supported by other groups, which conducted the factor analysis[4-6] .

Simms et al[7] proposed another four-factor model in 2002. Their model includes re-experiencing, avoidance, dysphoria, and hyper-arousal. The difference between their model and King's model is that they separated hyper-arousal symptoms into a larger dysphoria factor as a non-specific PTSD component, representing a general level of distress[6-12]. It is possible that different models can be used for analyzing different data sets[6]. For example, the model proposed by Simms et al[7] may be useful for analysis of the PTSD Checklist (PCL) data[13], while the model proposed by King et al[3] may analyze the Clinician Administered PTSD Scale[14]. Currently, it is still a challenge to determine the symptom structure of PTSD in the DMS system.

The current PTSD criteria miss several reactions that many trauma survivors experience. There is a new study suggesting that the current diagnostic procedures for PTSD are insufficient[15]. The study believes that some items need to be added in the current criteria including the nature of a traumatic event to reflect the relevancy of an individual's subjective experience in determining what constitutes a traumatic event[15] and claims that both objective and subjective factors are relevant. They report that individuals adapt to extreme experiences in a highly complex and coordinated manner. Trauma response is multifaceted and includes appraisals and thoughts, emotions and behaviors. However, it is difficult to quantify an experience whether it is traumatic objectively. Individual variation is

induced by many factors. Thus, trauma or not trauma is an individual matter, interaction between the individual and his or her environment, and all parts of an individual's response. Since PTSD is a traumatic event associated disease, understanding how to define a traumatic experience is critical. It is suggested to add more appropriate criteria to more accurately categorize traumatic events. Knowing exactly what trauma is can help us to better understand who is a trauma survivor and who is not. Therefore, it is critical to keep this in our mind for the purposes of understanding the disorder and resilience, which has been shown in those who are survivors of trauma.

5. Research of quantification of PTSD symptoms

The PTSD diagnostic criteria have been revised several times in the DSM system. The current diagnostic criterion for PTSD is the DSM system [16]. In the DSM-IV-TR (2000), the diagnostic criteria (A-F) are specified below. Diagnostic criteria of DSM-IV-TR for PTSD include a history of exposure to a traumatic event, meeting two criteria and symptoms from each of three symptom clusters: intrusive recollections, avoidant/numbing symptoms, and hyper-arousal symptoms. A fifth criterion is duration of symptoms and a sixth assesses functioning.

5.1 The DSM-IV-TR[16]

Criterion A: stressor

The person has been exposed to a traumatic event in which both of the following have been present:

1. The person has experienced, witnessed, or been confronted with an event or events that involve actual or threatened death or serious injury, or a threat to the physical integrity of oneself or others.
2. The person's response involved intense fear, helplessness, or horror. Note: in children, it may be expressed instead by disorganized or agitated behavior.

Criterion B: intrusive recollection

The traumatic event is persistently re-experienced in at least one of the following ways:

1. Recurrent and intrusive distressing recollections of the event, including images, thoughts, or perceptions. Note: in young children, repetitive play may occur in which themes or aspects of the trauma are expressed.
2. Recurrent distressing dreams of the event. Note: in children, there may be frightening dreams without recognizable content.
3. Acting or feeling as if the traumatic event were recurring (includes a sense of reliving the experience, illusions, hallucinations, and dissociative flashback episodes, including those that occur upon awakening or when intoxicated). Note: in children, trauma-specific reenactment may occur.
4. Intense psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event.
5. Physiologic reactivity upon exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event.

Criterion C: avoidance/numbing

Persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness (not present before the trauma), as indicated by at least three of the following:

1. Efforts to avoid thoughts, feelings, or conversations associated with the trauma
2. Efforts to avoid activities, places, or people that arouse recollections of the trauma
3. Inability to recall an important aspect of the trauma
4. Markedly diminished interest or participation in significant activities
5. Feeling of detachment or estrangement from others
6. Restricted range of affect (e.g., unable to have loving feelings)
7. Sense of foreshortened future (e.g., does not expect to have a career, marriage, children, or a normal life span).

Criterion D: hyper-arousal

Persistent symptoms of increasing arousal (not present before the trauma), indicated by at least two of the following:

1. Difficulty falling or staying asleep
2. Irritability or outbursts of anger
3. Difficulty concentrating
4. Hyper-vigilance
5. Exaggerated startle response

Criterion E: duration

Duration of the disturbance (symptoms in B, C, and D) is more than one month.

Criterion F: functional significance

The disturbance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Specify if:

Acute: if duration of symptoms is less than three months

Chronic: if duration of symptoms is three months or more

Specify if:

With or without delay onset: Onset of symptoms at least six months after the stressor

In the DSM system, the "A" stressor criterion specifies that a person has been exposed to a catastrophic event involving actual or threatened death or injury, or a threat to the physical integrity. Subjects experience intense fear, helplessness, or horror, during the traumatic exposure. In the "E" stressor criterion, it is duration, which qualifies for the (chronic or delayed) PTSD diagnosis. For example, in DSM-III, the mandatory duration is six months. In DSM-III-R, the duration is one month, where it has remained in DSM-IV. Finally, the functional significance criterion (F) indicates that the subject must have significant social, occupational, or other distress as a result of these symptoms.

Since 1980, the first time the diagnosis of PTSD appeared in the DSM system, many diagnostic tools have been developed. For example, working with Vietnam War zone veterans, both psychometric and psychophysiology assessing techniques were developed. In addition, modified assessment instruments have been used in many research subjects, such as the natural disaster victims, rape/incest survivors, subjects of Sept. 11 and the military personnel who deployed to Iraq and Afghanistan wars.

Several methods have been used in quantification and assessing of PTSD symptoms, including the Clinician-Administered PTSD Scale (CAPS)[14] and the PTSD Checklist (PCL)[13]. The CAPS is a structured interview for assessing PTSD (PTSD diagnostic status and symptom severity. In the last 20 years, since it was developed at the National Center for PTSD, the CAPS has become a commonly used criterion to determine PTSD and its

symptom severity. More than 200 studies used it, indicating its excellent reliability, yielding consistent scores across items, raters, and testing occasions. It also has excellent convergent and discriminate validity, diagnostic utility, and sensitivity to clinical change. The CAPS has been used in many pharmacological treatment studies of PTSD, and modified as CAPS-2. Using CAPS-2 to examine the therapeutic response, Nagy et al[17] and van der Kolk et al[18] report that fluoxetine significantly decreased CAPS-2 total score. Katz et al[19] and Baker et al[20] also use the CAPS to examine patients' response to brofaromine. Hertzberg et al[21] and Busuttil et al[22] used the CAPS to examine inpatient group therapy. However, there are several concerns about the CAPS. First, it may take longer on paper than other PTSD interviews. Second, it may be complicated and not easy to learn. Finally, its frequency and intensity ratings overlap. The CAPS has been revised several times with the most significant revision occurring after the publication of the DSM-IV in 1994. It is suggested that CAPS diagnostic ability would be enhanced by incorporation of a multimodal assessment. Currently, in general, CAPS has been used as a screening instrument and as a self-report measure of degree of post-traumatic stress symptoms[23].

Several other self-report inventories, such as Foa[24] and Davidson's Self-Rating PTSD Scale[25] and the older Impact of Events Scale[26] also have been used in PTSD research. Among them, Impact of Events Scale - Revised (IES-R) has been commonly used in research. IES-R is a 22-item self-report measure to assess subjective distress caused by traumatic events[26]. It contains seven additional items related to the hyper-arousal symptoms of PTSD. Its items correspond to 14 of the 17 DSM-IV symptoms of PTSD. It is to identify a specific stressful life event and then indicate how much the patient was distressed or bothered during the past seven days. Each of items is rated on a five-point scale ranging from 0 ("not at all") to 4 ("extremely"). The total score (ranging from 0 to 88) and subscale scores can also be calculated for the intrusion, avoidance, and hyper-arousal subscales. It is suggested to use means instead of raw sums for each of these subscales scores to allow comparison with scores from the Symptom Checklist 90 – Revised (SCL-90-R)[27]. While the IES-R is not used to diagnosis PTSD, it is reported that its cutoff scores can be applied in a preliminary diagnosis of PTSD.

6. Brain imaging and magnetoencephalography (MEG) in PTSD research

Brain image, such as functional magnetic resonance imaging (fMRI), has enhanced our ability to examine the structural and functional properties of the brain in PTSD. Several lines of evidences demonstrate that fMRI and magnetoencephalography (MEG) can be used for PTSD diagnosis and monitoring therapeutic responses. In an fMRI study, Yin et al report that PTSD patients show decreased amplitude of low-frequency fluctuation (ALFF) values in right lingual gyrus, cuneus, middle occipital gyrus, insula, and cerebellum, and increased ALFF values in right medial and middle frontal gyri, relative to traumatized individuals without PTSD[28]. The ALFF value in the right medial frontal gyrus is positively correlated with severity of the disorder. To examine how the function of brain changes during the recovery from PTSD, PTSD patients underwent two fMRI scans, 6-9 months apart, while viewing fearful and neutral faces in preparation for a memory test (administered outside the scanner). At the second scan, 65% of patients were in remission and their current symptom levels correlated positively with memory-related fMRI activity in the amygdala and ventral-medial prefrontal cortex (vmPFC). The change in activity of hippocampus and the subgenual anterior cingulate cortex (sgACC) is associated with the

degree of symptom improvement. These data indicate that differential brain regions within the fear network play different roles in symptom manifestation and in recovery from PTSD. The amygdala and vmPFC appear to be specific brain regions having the activity to serve as a marker for current symptom severity, while the functional changes in the hippocampus and sgACC may be a marker for recovery[29]. Meanwhile it is also found that there is greater recruitment and coupling of emotional brain regions during the retrieval of negatively intense autobiographical memory in the PTSD group when compared to controls[30]. PTSD patients have less activation to the threat condition and increased activity to the safe condition in the subgenual cingulate, ventral striatum and extended amygdala, as well as in midbrain periaquaeductal grey. These data indicate abnormal reactivity in these key regions for fear expression. The temporal pattern of activity decrease found in control subjects was not obtained in PTSD patients[31]. Imaging analyses also find decrease of activity in the amygdala and hippocampus of PTSD patients during successful encoding of trauma-related stimuli. Such decrease in left hippocampus is associated with high arousal symptoms. These results indicate reduction of hippocampal activity under conditions of high stress and arousal[32]. Significant improvements of PTSD are evident on fMRI scans, and corroborated by Clinical Global Impression (CGI) scores, but CAPS scores improvements are modest, indicating CGI scores and fMRI scans can be used to examine the improvement of PTSD.

Patients with child abuse-related complex PTSD show reductions in gray matter levels in right hippocampus, right dorsal ACC and the right orbitofrontal cortex (OFC) compared to controls. Meanwhile their child abuse and hyper-arousal correlated negatively with ACC volume. Impulsivity and anger correlated negatively with hippocampus volume, and OFC volume respectively. In another study, it is found that PTSD is associated with smaller mean cornu ammonis 3 (CA3)/dentate gyrus subfield volumes and total hippocampal volume, indicating that PTSD is associated CA3 implying that chronic stress suppresses neurogenesis and dendritic branching in this structure[33]. As in adult PTSD, children with symptoms of post-traumatic stress suffer poor function of the hippocampus, exhibiting more errors on the recall part of the test and showing less hippocampus activity than control subjects doing the same task[34].

MEG, a test that measures magnetic fluctuations faster as groups of neurons fire (neither CT scans nor MRIs can measure it), has been used for PTSD diagnosis. The MEG is cutting edge, but it's not new technology. It has been used since the late 1960s. Perhaps best of all, the mapping of synchronous neural interactions (SNI) works regardless of what the person being observed is doing. Recently one study shows that PTSD patients demonstrate a unique pattern of miscommunication. The PTSD patients show impaired. A 50-ms response (M50) gating in the right hemisphere. Thinner right superior temporal gyrus (STG) cortical thickness is associated with worse right sensory gating in the PTSD subjects. The right primary sensory cortex (S1) M50 source strength and gating ratio is correlated with PTSD symptomatology. These findings indicate that the structural integrity of right hemisphere STG cortices play an important role in auditory sensory gating deficits in PTSD[35]. It is found that the SNI test which assesses the functional interactions among neural populations derived from MEG recordings[36] can successfully differentiate PTSD patients from healthy control subjects.

7. Complications of PTSD

Individuals with PTSD are 8–14 times more likely to have a second lifetime diagnosis of psychosis after the development of PTSD, with 50–80% of those being affective and anxiety

disorders[37]. PTSD is more common among depressed primary care patients than previously thought, and 62% of individuals diagnosed with PTSD have suicidal ideation[38]. Here we discuss several common mental disorders seen in PTSD patients, such as alcohol and drug abuse, and depression.

7.1 PTSD and alcohol and drug abuse

People with PTSD are more likely than others of similar background to have alcohol use disorders both before and after being diagnosed with PTSD. For example, 25-75% and 10-33%of survivors of abusive or violent trauma, accidental illness, or disaster trauma have problematic alcohol use, respectively. 60-80% of Vietnam veterans seeking PTSD treatment have alcohol use disorders. Veterans over the age of 65 with PTSD have increased risk for attempted suicide if they experience problematic alcohol use or depression. Those survivors with alcohol problems have ongoing health problems or pain. In addition, there is gender difference in drinking. Women who go through trauma have more risk for drinking problems. 27.9% of women with a history of PTSD report problems with alcohol abuse or dependence at some point in their Lifetime. Almost twice as many men (51.9%) with a history of PTSD reported such problems. As a point of comparison, Kessler and colleagues find that, on average, 24.75% of men and 10.55% of women without PTSD have problems with alcohol or drugs at some point in their lifetime. Alcohol use and intoxication, and suddenly stopping drinking can result in some symptoms worse, including avoidance, anger, irritability and depression. Suddenly stop drinking can also result in worsening of some the symptoms. In addition to alcohol, the consistent findings demonstrate that patients with PTSD have a high risk of drug use. For example, 34.5% of men and 26.9% of woman with PTSD also have a problem with drug abuse or dependence during their lifetime.

7.2 PTSD and depression

Depression is one of the most frequent comorbid conditions in individuals with PTSD. In fact, 48% of subjects with PTSD also have current or past depression. Individuals with PTSD are almost seven times as likely as individuals without PTSD to have depression. 44.5% of individuals with PTSD one month after experiencing a traumatic event have a diagnosis of depression[39-42].

7.3 PTSD and anxiety

Like PTSD and depression, there is a strong relationship between PTSD and anxiety disorders. Patients with PTSD are more likely to have anxiety disorders. Around 7% of patients with PTSD at some point in their life also have had a diagnosis of Panic Disorder. Patients with PTSD are four times as likely to also have a current or past diagnosis of Panic Disorder as compared to non-PTSD control. Approximately 28% of patients with PTSD have or have had a diagnosis of Social Anxiety Disorder. Patients with PTSD are three times as likely as someone without PTSD to have Social Anxiety Disorder. It has been found that 4% to 22% of patients with PTSD also have obsessive-compulsive disorder (OCD). Finally there are strong relationships between PTSD and other anxiety disorders, such as phobia. Over 30% of patients with PTSD had or have had a specific phobia. Patients with PTSD are seven times as likely as people without a history of PTSD to have also had a specific phobia.

7.4 PTSD and traumatic brain injury (TBI)

Since the war in Iraq and Afghanistan, PTSD and TBI were significantly increased in the military service members. PTSD and TBI are comorbid. Patients with both PTSD and TBI exhibit a spectrum of common clinical features such as difficulty in concentrating, sleep disturbance, depression, anxiety, irritability, fatigue, suicidality, chronic pain, and alterations in arousal. PTSD and TBI disorders have overlapping neural mechanisms with changes in hippocampal, prefrontal cortical and limbic region function because of alterations in synaptogenesis, dendritic remodeling, and neurogenesis. These neural changes in PTSD and TBI may result from pathophysiological disturbances in metabolic, cytotoxic, inflammatory, and apoptotic processes, amongst other mechanisms[43].

7.5 PTSD and metabolic syndrome

Several lines of research suggest that stress and post-stress adaptation responses are related to long-term health outcomes. Studies of survivors of disasters, veterans and prisoners of war, and others exposed to severe trauma, suggest higher rates of physical morbidity and mortality and increased healthcare utilization related to lifetime prevalence of trauma. Accumulating evidence from epidemiological studies demonstrate that chronic PTSD associated with trauma and secondary negative health outcomes including metabolic conditions. 43% of PTSD met criteria for metabolic syndrome, indicating that PTSD is associated with risk factors for diminished health and increased morbidity, as represented by metabolic syndrome.

7.6 PTSD and pain

The co-occurrence of PTSD and chronic pain symptoms have been observed clinically. For example, it is found that 10% of patients referred to a Veterans Administration pain clinic meet criteria for PTSD and that 9.5% of patients attending a multidisciplinary chronic pain center meet criteria for "post-traumatic pain syndrome"[44]. In the patients who are referred for the assessment of a chronic pain problem resulting from a traumatic event, the prevalence of PTSD is also high. Hospitalized burn patients have high rates of PTSD at 12 months post-injury[45]. 80% of PTSD patients report the presence of a chronic pain condition. In addition, PTSD re-experiencing symptoms were positively associated with pain level and pain-related disability. The co-occurrence of chronic pain and PTSD may have implications in terms of an individual's experience of both conditions. Patients with chronic pain related to trauma or PTSD experience more intense pain and affective distress[46], higher levels of life interference[47], and greater disability than pain patients without trauma or PTSD[48, 49]. The most common forms of chronic pain for survivors of trauma are pain in the pelvis, lower back, face and bladder; and in fibromyalgia; interstitial cystitis; and non-remitting Whiplash syndromes. However, the relationship between PTSD and chronic pain is not always noticed and is often overlooked[50, 51]. Both PTSD and chronic pain can increase the symptom severity of either condition[52]. Patients with chronic pain may focus their attention toward their pain while individuals with PTSD may unknowingly focus on things that remind them of the trauma. Consequently, both PTSD and chronic pain may result in patients having less time and energy to adapt their pain and fear. Furthermore, patients with PTSD often have hyper-arousal and tension, which may interference their perception of pain. Patients with co-morbid pain and PTSD demonstrate more intense pain, more emotional distress, higher

levels of life interference, and greater disability than pain patients without PTSD. Treating these patients can also be more complex and challenging.
Currently, the mechanism for the co-occurrence of PTSD and pain is still unknown. There are several theoretical models, such as mutual maintenance model[51], shared vulnerability model[50], fear-avoidance model and triple vulnerability model[53]. However, those models needed to be tested.

7.7 PTSD and other diseases

The association of traumatic life events with PTSD and other health conditions is well known. For example, patients with PTSD had substantially higher (i.e., 50–150% greater) postwar rates of many major chronic diseases, including circulatory, nervous system, digestive, musculoskeletal, and respiratory diseases[54]. PTSD is significantly associated with an almost two-fold increase of developing nervous system, musculoskeletal disease, and signs and ill-defined conditions of disease. PTSD is significantly associated with increased odds of developing circulatory, hypertensive, and digestive system disease[55]. More data shows that PTSD patients have abnormally high white blood cell counts (>11,000/mm3) and T-cell counts (>2,640/mm^3)[56].
Evidence linking exposure to traumatic stress and cardiovascular disease is compelling. 25% of PTSD veterans report physician-diagnosed circulatory diseases (vs. 13% for PTSD negative veteransIt is also reported that PTSD veterans are significantly more likely to have had abnormal electrocardiograph (ECG) results (28% vs. 14%) with a higher prevalence of myocardial infarctions and atrioventricular conduction defects[57]. There are positive association between chronic PTSD and myocardial infarction (MI) as well as lower plasma levels of high-density lipoprotein-cholesterol (HDL-C)[54, 57, 58]. In a population study involving World War II and Korean War veterans, it is found higher rates of physician-diagnosed cardiovascular disease among PTSD subjects[59]. Another study among Dutch resistance fighters demonstrate increased rates of cardiovascular disease risk factors among PTSD[60]. A large-scale civilian population study also found an increase in ischemic heart disease among adults exposed to childhood traumas[61]. Furthermore, adults exposed to the Chernobyl disaster have increased rates of reported heart disease[62]. In addition, studies during the Beirut Civil War and the Croatia War find increases in arteriographically confirmed coronary heart disease, cardiovascular disease mortality, and increases in acute myocardial infarction (AMI) associated with exposure to these conflicts[63, 64]. An increase in AMIs is also reported after the Hanshin-Awaki earthquake in Japan[64].
Evidence indicates that exposure to environmental stressors and subsequent development of PTSD may be related to altered neuroendocrine and immune system functions, and the onset of specific immunoendocrine-related diseases. Either increases or decreases of circulating cortisol levels in PTSD patients are reported. The former indicates an acute response or up-regulated glucocorticoid system, while later suggesting a chronic or downregulated glucocorticoid system. Either direction of these changes may result in an alteration activities of immune inflammatory. Indeed. glucocorticoids influence the trafficking of circulating leukocytes and affect functions of leukocyte and immune accessory cells. Hyper-arousal is often observed during recollection of traumatic events by PTSD victims and is associated with alterations in the neuroendocrine functions. Although these processes are complex, chronicity and excessiveness of stress system activation in PTSD could be one possible pathogenesis, which is associated with weight loss, depression,

hypogonadism, immunosuppression, and other pathophysiological conditions leading to many diseases. Therefore, the research in these specific areas is demanded and emphasized to improve the quality of life of patient with PTSD[65].

8. Treatment of PTSD

There are many therapeutic methods for treatment of PTSD, including cognitive-behavioral therapy (CBT) and medication. In clinical study, it is reported that combinations of exposure therapy and cognitive restructuring as well as CBT produces better therapeutic response, especially in the treatment of female victims of childhood or adult sexual trauma.

The first FDA approved group of compounds for PTSD are selective serotonin reuptake inhibitors (SSRIs), including sertraline (Zoloft) and paroxetine (Paxil). In addition, there is a report showing that Eye Movement Desensitization and Reprocessing (EMDR) may have better therapeutic response as well.

Dr. Friedman suggests that mildly to moderately affected PTSD patients may need group therapy (http://www.veterans.gc.ca/eng/sub.cfm?source=mental-health/support/factsshc). In this therapeutic approach, the PTSD patient is asked to discuss their traumatic memories, PTSD symptoms, and functional deficits. However, since PTSD is a chronic and severely debilitating psychiatric disorder, there is no better approach yet. New ideas and approaches need to be developed. The following is a brief summary of current available approaches for treatment of PTSD.

Currently, several psychotherapies have been used in clinical practice, including CBT, exposure therapy, stress inoculation training (SIT), cognitive restructuring, and EMDR. CBT has been shown to be the most effective type of therapeutic treatment. CBT helps to recognize and change inaccurate thoughts about subjects. Exposure therapy is the best technique for recovery, involving overcoming anxieties by facing them in a controlled and safe environment and relieving fears all at once (flooding) or step-by-step (desensitization) in order to overcome them. Although this may seem frightening at first, this treatment produces a quick outcome. Supportive counseling, without facing the trauma, has also been shown to be helpful, but may not be as effective as direct exposure. The SIT consists of teaching the PTSD subjects to manage their anxiety reactions to situations, memories, etc. they normally fear and avoid. For the physical manifestations of anxiety (heart rate, hyperventilation, and muscle tension). The SIT teaches controlled breathing and progressive muscle relaxation. For intrusive thoughts and worrying, The SIT teaches patients how to interrupt their thought patterns and think of positive imagery. By this way, the PTSD can control and lessen their PTSD symptoms.

The other therapy is cognitive restructuring, which helps subjects identify and challenge their erroneous beliefs and interpretations. It is based on the idea that it is not actual events that cause negative emotional reaction but the interpretation of those events, letting to the replace worry and anxiety with more positive and productive emotions.

In addition, EMDR allows the therapist to have the PTSD patients remember their trauma briefly and then engage in cognitive restructuring.

In recent years, several alternative therapies have been introduced in PTSD treatment, although its effectiveness is still need to be determined. They are included in massage, acupuncture, art and music therapy, drama therapy and exercise.

8.1 Prognosis

The best outcome, or prognosis, depends on how soon the symptoms develop after the trauma, and on how quickly the patient is diagnosed and treated.

9. Biomarker research in PTSD

The term biomarker (biological marker) was first described in 1989 as a substance used as an indicator of a biological state. It refers to a Medical Subject Heading term: ''measurable and quantifiable biological parameters (e.g., specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiologic studies, etc". In 2001, a definition of a biomarker was described by an NIH working group as ''a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (NIH Biomarker Definitions Working Group, 2001). In 2004, FDA announced that quantitative measures of biological effects that provide informative links between mechanism of action and clinical effectiveness are considered to be biomarkers (FDA whitepaper 'Innovation or Stagnation' 2004). The World Health Organization (WHO) defines the biomarker as any substance, structure or process that can be measured in the body or its products and can influence or predict the incidence or outcome of disease (WHO International Program on Chemical Safety).

Biomarker researches progressed significantly during the 1990s. Since then, the whole field of biomarker research has been drastically changed by the human genome project. The information gained from the human genome project redirected biomarker studies into a whole new era. High-throughput analytical instruments have been used to screen thousands of genes and proteins simultaneously. Today, biomarkers have been classified into four subgroups including type 0 biomarker, type 1 biomarker, surrogate end point-type (type 2 biomarker) and risk marker.

Type 0 biomarker: A marker of the natural history of a disease and correlates longitudinally with known clinical indices.
Type I biomarker: A marker that captures the effects of a therapeutic intervention in accordance with its mechanism of action.
Surrogate end point (type 2 biomarker): A marker that is intended to substitute for a clinical end point; a surrogate end point is expected to predict clinical benefit (or harm or lack of benefit or harm) on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence.
Risk marker. For prevention, the important disease risk factors are those that can be measured quantitatively in the subject at risk. These factors can be used to identify cohorts for prevention. Those may also be used as endpoints in prevention studies.

Table 1. Identification of Biomarkers

9.1 Potential biomarker(s) for PTSD

High-throughput "omic" approaches including genomics, proteomics, and metabolomics have been used in candidate biomarker selection and identification for many diseases[66] (Table 2). However, progress of the search for PTSD biomarkers has been slow and often frustrating because the complexity of the molecular mechanisms of the disease and the undefined validation process. Potential biomarker(s) for PTSD on the basis of animal research[67] or limited studies in humans have been proposed. But confirmation and validation of their clinical utility have not been accomplished. Appropriate and efficient validation would be expedited with valid animal models, standardized laboratory practice, larger human population-based studies, and repeated sampling of individuals. A strategy used in biomarker development for other illnesses[68] can also be used for development of a blood biomarker test for PTSD. Here, we will discuss the strategy including screening approach as well as analytical and clinical validations to facilitate PTSD blood biomarker discovery and selection.

Technology	Method	Test
Genomics	SNP genotyping	Susceptibility or disease modifying gene
	Positional cloning/microsatellites	Fine mapping/sequencing of disease loci
	Microarray	Gene expression
Proteomics	2DGE, MS, LC-MS, GC-MS, MS-MS, MALDI-TOF MS	Protein expression
Metabolomics	NMR spectroscopy, MS, infrared spectroscopy	Small molecule

Table 2. Biological tools are used in screening PTSD biomarker

In the case of PTSD, to screen the potential biomarkers, researchers currently compare the gene and protein expression profiles or alteration of a gene, protein expression or metabolite levels between PTSD patients and healthy control subjects by using several well developed procedures (Table 1). Biomarkers may be obtained from saliva, blood, cerebral spinal cord fluid, urine and tissues. It also can be physiological parameters such as blood pressure[69], ECG[70], and hart betting[71], transmitters, such as 5-HT[72], dopamine[73] and GABA[74] or their metabolites, and brain-imaging[75]. Biomarkers indicate PTSD or PTSD characteristics, including the level or type response of exposure to a traumatic stress, genetic susceptibility, genetic responses to traumatic stress exposure, markers of subclinical or clinical state, or indicators of response to therapy. These markers may dynamically alter during the course of PTSD development or differentially change after single or multiple traumatic stresses, respectively (Table 3).

Potential biomarker	References
T cell phenotypes	Lemieux A, Coe CL, Carnes M. 2008 [76]
Assumptions	Rosen GM, Lilienfeld SO. 2008 [77]
Erythrocyte sedimentation rate, white blood cell count, and cortisol/ dehydroepiandrosterone-sulfate ratio	Boscarino JA 2008 [78]
Endothelial dysfunction in plasma	von Känel R, 2008 [79]
Serum interleukin-2 and interleukin-8 levels	Song et al. 2007 [80]
Platelet serotonin concentration	Kovacic Z et al., 2008 [72] Pivac N, et al., 2006 [81] Mück-Seler D, et al., 2003 [82] Spivak B, et al., 1999 [83]
Platelet MAO-B activity	Pivac, N et al., 2007 [84]
Circulating Cortisol levels	Meewisse ML et al., 2007 [85] Ehlert U et al., 2001 [86] Heber R, et al., 2002 [87] Glover DA, Poland RE, et al., 2002 [88] Yehuda R, et al., 2002 [89]
Glucocorticoid receptor (GCR) expression in lymphocyte	Gotovac K, et al., 2003 [90]
WFS1 gene	Kesner Y et al, 2007 [91]
Baseline level of platelet-leukocyte aggregates, platelet CD63 expression, and soluble P-selectin concentration	Vidović A et al., 2007 [92]
GABA plasma levels	Vaiva G et al.. 2006 [93]
S-100B and neuron-specific enolase	Sojka P et al., 2006 [94]
NPY expression	Dutton MA, Lee EW, Zukowska Z. 2006 [95]
Myelin basic protein	Wang Q, et al., 2004 [96]
C-reactive protein and serum amyloid A	Söndergaard HP, et al., 2004 [97]
Urinary dopamine	Glover DA, et al. 2003 [88]
Thyroid hormone	Garrison RL, Breeding PC. 2003 [98]
Neopterin	Atmaca M, et al., 2003 [99]
Plasma and cerebrospinal fluid interleukin-6 concentrations	Barker DG, et al., 2002 [100] Maes M, et al., 1999 [101]
REM latency	Reist C, et al., 1995 [102] Kauffman CD et al, 1987 [103]
Average heart rate responses to a series of sudden, loud-tone presentations	Pitman RK, et al., 2006 [104] Bryant RA. Et al., 2007 [105]
Mixed lateral preference and parental left-handedness	Chemtob CM, Taylor KB, 2003 [106]
Startle responses	Milde AM, et al., 2003 [107]

Table 3. Potential Biomarkers for PTSD

PTSD biomarkers can be the indicators of PTSD trait (risk factor or risk marker), disease state (preclinical or clinical), or disease rate (progression). The PTSD biomarkers in all possible respects: antecedent, screening, diagnostic, staging and prognosis are listed in Table 4.

Biomarkers	Function or indicator
Antecedent	Identifying the risk of developing PTSD, changes in response to single or multiple traumatic stress events
Screening	Subclinical PTSD from well adapted subjects, physiological responding
Diagnostic	Recognizing PTSD
Staging	Categorizing PTSD severity
Prognostic	Predicting PTSD course: recurrence and response to treatment

Table 4. Types of biomarker for PTSD

PTSD biomarker(s) may prove to be a single biological parameter associated with a highly specific symptom or a multiple biological indicator (cluster) related to multiple symptoms. Individual biomarkers have been shown to be a significant predictor of PTSD occurrences. Few studies have evaluated the use of multiple biomarkers for patient risk stratification[67]. The assessment of multiple biomarkers[67] may be useful for identifying subgroups of PTSD that would benefit most from additional testing.

The desirable properties of biomarkers for PTSD vary with their different usage. For example, a screening test may be used in large populations including normal and high-risk subjects. Thus, the biomarker requires having high sensitivity, specificity, and predictive values, large likelihood ratios, and low cost. A diagnostic biomarker should be appropriate for use at any stages of the disease (acute and chronic) and have consistent detectability in patients at any stage of illness or treatment. For biomarkers to be used in monitoring PTSD progression or response to therapy, features such as sensitivity or specificity may be less important because the patient serves as his or her own control (baseline values are compared with follow-up values). The ability to monitor intra-individual variation as this relates to clinical status is more important. Costs may be less important for prognostic markers since only people with PTSD will be tested. Searching for prognostic PTSD biomarkers may be most challenging because it requires a larger sample and prospective design, whereas a diagnostic biomarker test requires relatively smaller sample size and a cross-sectional design. While all the features of biomarkers can be combined in one for a screening test, having specific features for specific use is the ultimate goal in the search for PTSD biomarkers. It would be ideal to have a biomarker that can differentiate the three conditions (physiological response, well adapted and acute PTSD) in the early traumatic stages. The overall benefit of a PTSD biomarker would be to enhance the ability of the clinician to optimally manage the patient with PTSD, and to help the researcher and clinician identify specific therapeutic targets. For instance, an EEG test may be expected to facilitate the identification of patients with sleep problems. A biomarker such as the level of glucocorticoid[108] and/or concentration of epinephrine in the blood may help to differentiate subjects who have transient acute response to traumatic stress from patients who ultimately

develop PTSD. A plasma GABA level may represent a marker of recovery from trauma[93]. In general, a biomarker test for PTSD should be not only accurate, reproducible, and standardized, but also acceptable to PTSD patients and easy-to-use for clinicians.

9.2 A strategy to identify a PTSD biomarker

Like studies of biomarkers in other medical fields, the strategy for identification of a PTSD biomarker includes three major steps: screening, analytical validation and clinical validation (Fig 1).

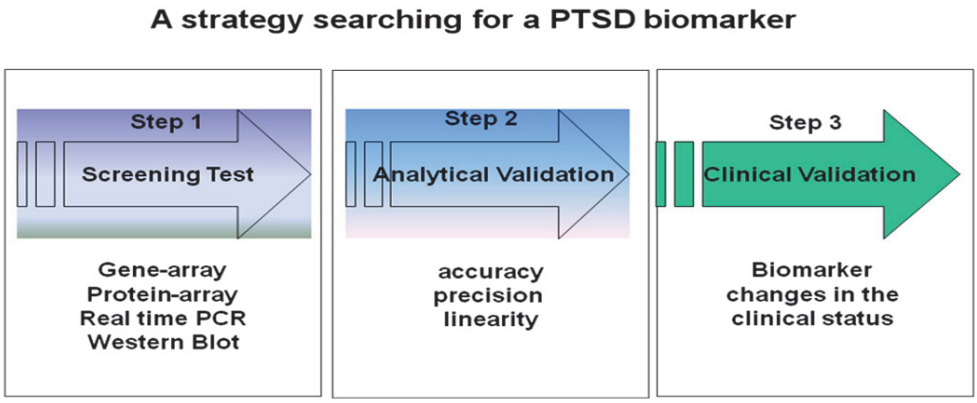

Fig. 1. A strategy searching for a PTSD biomarker

9.2.1 Screening a biomarker(s) for PTSD

Currently, many biological tools have been used for screening potential PTSD biomarkers (Table 2). Biomarker screening is the first step in identifying a potential PTSD biomarker. During the screening stage, animal models have been used. However, the complexity and variability of PTSD symptoms make the establishment of generally validated animal models for biomarker screening more challenge[67]. The most current animal models use different stress paradigms to provoke a wide range of behavioral responses. In addition, results have been presented as mean values (with SD or SE) of the entire exposed population. These animal models may overlook the clinical finding that only a proportion of individuals (20%–30%) who are exposed to a "traumatic event" will eventually develop PTSD[67]. Therefore, multi-dimensional validation, such as a combination of both behavioral and biological criteria, may be necessary to provide a solid basis for the biomarker screening study and for the molecular mechanism research[67].

Compared to other psychiatric and medical illnesses relatively little work has been directed toward elucidating the molecular mechanisms of posttraumatic psychopathology. There are some association studies for individual molecular targets, but these are inherently limited to hypothesis–driven search for candidates already implicated through the current framework of biological theorizing. Previously, the possibility of identifying molecular targets for PTSD in a genome-wide screen in a validated animal model using microarray gene expression profile analysis have been discussed[67]. The mRNA levels of p11, a member of the S-100 protein family, increase in the post mortem prefrontal cortex (area 46) of PTSD patients[109]. Stress, induced by three days of inescapable shock, increases both p11 mRNA levels in the prefrontal cortex (PFC)

of rats and corticosterone levels in plasma. Dexamethasone (Dex), a synthetic glucocorticoid, up-regulates p11 expression in SH-SY5Y cells through glucocorticoid response elements (GREs) within the p11 promoter, which can be attenuated by either a glucocorticoid receptor antagonist, RU486, or mutating two of the three glucocorticoid response elements (GRE2 and GRE3) in the p11 promoter. This work demonstrates not only an example of a study of mechanisms of posttraumatic psychopathology, but also identification of a potential PTSD biomarker, p11. Also, mitochondrial gene expression profiling in post-mortem brain of patients with PTSD[110] serves as a source of biomarkers. The study emphasizes the possible molecular mechanisms for PTSD, the analytical validation of sample purity, large scale scanning and cluster classification. Obtaining pure samples for genomic analysis requires highly stringent criteria and should be validated along three dimensions—analogous (similarity of behavior), predictive (predictability of drug response) and biological mechanism (gene expression). Analytical validation should also be considered.

9.2.2 Analytical validation for a PTSD biomarker(s)

Although several candidate biomarkers for PTSD have been identified by screening approaches, few have been validated[81, 93, 108, 111]. Meaningful validation requires high-throughput bioinformatics and large sets of data. In addition to statistical validation and biological/functional validation, analytical validations may be more important and have to be completed prior to their use in clinical sites. In 1999, the United States Food and Drug Administration established a final guidance for the industry validation of analytical procedures and terminology[112,113]. This document provides guidance on characteristics for consideration during the validation of analytical procedures. This guidance represents current thinking about validation. According to this guideline, analytical validation include the following aspects: precision (reproducibility), accuracy (clinical samples; compare to cleared or gold standard method), limit of detection, potential interferences software, sample preparation/conditions, performance around the cut-off, potential for carryover or cross-hybridization and assay limitations. Obviously, the guideline can be used for analytical validation of PTSD biomarkers. In this guideline many concepts or terms were defined. We review several major terms related to PTSD biomarker development here. First is **precision**. In the guideline, precision was defined as reproducibility, which requires that intended users using clinical samples and all analytical steps of the assay get reliable results; User training should be the same for studies and for marketed assay. Precision is considered at three major levels: repeatability, intermediate precision and reproducibility. Repeatability expresses the precision under the same operating conditions over a short interval of time and is also termed intra-assay precision. Intermediate precision expresses within laboratory variations (different days, different analysts, different equipment, etc). Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology) and is assessed by means of an inter-laboratory trial.

The second term that we discuss here is **accuracy**. The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value. Accuracy requires the use of real clinical samples; to compare to a reference method; in limited cases (i.e., very rare alleles) contrived samples can be used; samples should mimic the molecular composition and concentration of real clinical samples. This is sometimes termed 'trueness'.

The third term is the **detection limit**. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at

which the analyte can be reliably detected. The detection limit of an analytical procedure is the lowest value of analyte in a sample. The maker can be detected but not necessarily quantitated as an exact value. Visual evaluation can be used for non-instrumental methods but may also be used with instrumental methods. Signal-to-noise with-this approach can only be applied to analytical procedures which exhibit baseline noise. The signal-to-noise ratio can be determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

It is also noticed that in the screening stage, microarray has emerged as an important format for simultaneous analysis of tens of thousands of substances present in a sample. Successful adaptation of microarray assays to clinical diagnostics will require particular attention to issues of quality control and quality assurance. Results of an assay can be compromised by a number of pre-analytical factors including the quality of the reagents (e.g., the microarray and the detection reagents) and the integrity of the sample. Similarly, numerous factors in the analytical phase of a microarray assay may compromise results, including changes in the reaction conditions and calibration. Furthermore, a microarray study combines many reagents or samples in a single device. This process brings additional confounds not usually encountered in discrete testing of a single analysis in a single sample. Thus, various strategies, such as replicate analysis and normalization have to be implemented to control and assess analytical factors in these studies. The current range of measures taken to ensure the analytical accuracy and quality of data generated from proteomics and metabolomics assays in other fields may also be applied in the context of DNA microarrays. Taking everything together, the data have to be documented with analytical validation for each of the operator and instrument prior to clinical validation.

9.2.3 Clinical validation for PTSD biomarkers

As a newly discovered biomarker assay makes the transition from a research setting to the clinical diagnostic laboratory, it should progress through defined stages of assay confirmation. At the screening stage, a validation focused on evaluation of research assay technology, performance, and specifications (analytical validation) is most important. However, the ultimate goal is validation of the test ability to identify PTSD. Assays have to be developed into final procedures that are standardized and reproducible for clinical diagnostic implementation. Thus, the final step of development of PTSD biomarker should be clinical validation (clinical utility).

Clinical validation or clinical utility will be based on new clinical trial data. These clinical data should be prospectively collected in a longitudinal study with appropriate institutional review board (IRB) and informed consent. These clinical samples must be well characterized. They will show clinical utility in prospective clinical studies and retrospective validation. Clinical and analytical cut-off points should be described and independently validated. Clinical cut-off points should be identified in a training set and validated in a test set. Clinical cut-offs can reference data from literature. Bridging studies are required if a platform change or device change is necessary after clinical validation.

For markers of clinical status, clinical validation refers to measurement of how biomarker level is related to a change in the clinical status of a patient[114]. The changes are not compared only with the average normal, but to the patient's own baseline values obtained when they are first diagnosed with the same procedure. The dynamic changes of the biomarker value in the same patient indicate the inherent variability of the data. The variability can be due in

part to the reproducibility of the analytical measurement, but also due to natural physiological changes or PTSD progression. Therefore longitudinal study of a control (healthy) population may be needed to determine the extent to which level changes represent biological variability rather than changes in disease status. In addition, to determine the relationship of biomarker alteration between peripheral and contral nervous system (CNS) could be more important in clinical validation study for PTSD. We found that biomarkers in the brain and in the blood are not necessarily altered in the same direction. Finally, the validity of the biomarker test, including its rates of false negatives and false positives should be well-established before the tests enter clinical use.

Currently, the diagnosis for PTSD is based on a certain set of symptoms determined from the patient's clinical history, mental status examination, duration of symptoms, and clinician administered symptom checklists or the patient self-report. However, there are no available laboratory biomarker tests for PTSD. To begin intervention at the earliest possible time, priority must be given to developing objective approaches to determine the presence of PTSD. Thus, a simple blood test or a biomarker that could detect PTSD in its earliest and potentially most treatable stages would be beneficial for physicians and patients. Currently, many potential biomarkers have been identified in animal models and in patients with PTSD. But those biomarkers have not been well validated. Current strategy to identify a biomarker for PTSD involves pre-clinical screening, analytical validations and clinical validations. This strategy will enhance not only the study of the molecular mechanisms of PTSD, but also the translation of basic science to clinical implications.

During the last decade, with the rise of genomics and advances in molecular biology, researchers have become increasingly focused on the underlying molecular mechanisms of PTSD and searching for a biomarker for PTSD. New technologies have the potential to identify PTSD biomarkers in blood to definitively diagnose patients with PTSD or to identify those who may be at high risk for developing PTSD after traumatic exposure. The development of such biomarker tests requires a strategy that involves pre-clinical screening, analytical validations and clinical validations, and is driven by the advances in research surrounding the underlying molecular mechanisms of PTSD (Fig 1).

The first potential PTSD biomarker has been discovered. In 2009, we reported that p11 mRNA expression is significantly changed in post mortem cortex of patients with PTSD and depression, and in their peripheral blood mononuclear cells (PBMC). We hypothesize that p11 mRNA levels in the peripheral blood cells can serve as a biomarker for PTSD with heterogeneity in terms of type of trauma, time since trauma and duration of illness. We examined the PBMC p11 mRNA of patients with PTSD (n = 13), major depressive disorder (MDD, n = 16), bipolar disorder (BP, n = 24), and schizophrenia (SCZ, n = 12) or controls (n = 14) using quantitative real-time PCR and the circulating levels of cortisol in blood plasma and saliva of PTSD patients using radioimmunoassay kit CORT-CT2. The Hamilton Rating Scale for Depression (HAMD) and Anxiety (HARS), the Chinese version of the Davidson Trauma Scale- Frequency (CDTS-F) and the Chinese version of the Davidson Trauma Scale-Severity (CDTS-S), and Impact of Event Scale-Revised (IES-R) were administered. We found that patients with PTSD had lower levels of p11 mRNA than control subjects, while those with MDD, BP and SCZ had significantly higher p11 levels than the controls. P11 mRNA levels were positively correlated with the scores of HAMD ($r = 0.62$, $p < 0.05$), CDTS-F ($r = 0.71$, $p < 0.05$) and CDTS-S ($r = 0.62$, $p < 0.05$), while they did not correlate with scores of HARS and IES-R. Basal levels of plasma and salivary cortisol of PTSD patients were not

statistically different from those of controls. Our findings suggest that PBMC p11 mRNA expression levels may serve as a biomarker to distinguish PTSD from BP, MDD and SCZ.

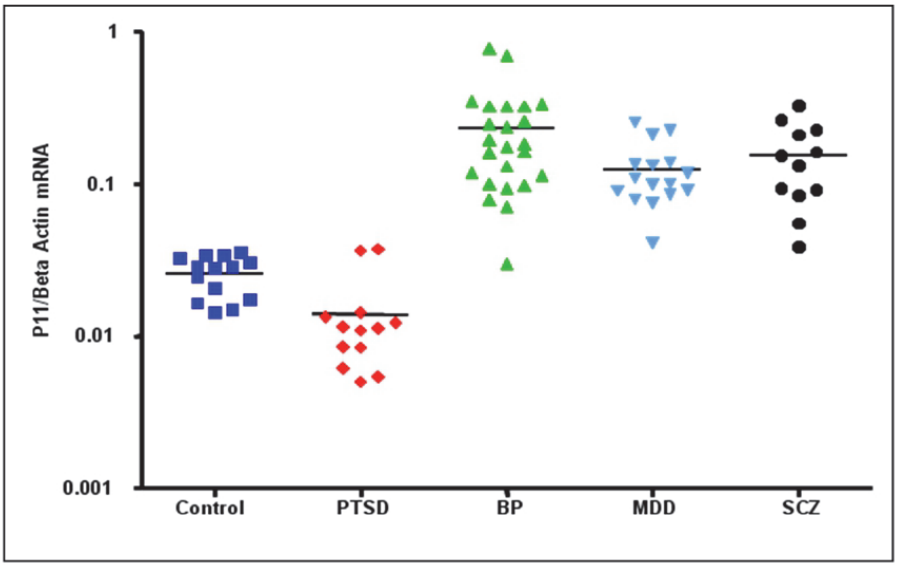

Fig. 2. Significantly lower p11 mRNA levels in the patients with PTSD compared to control and other mental disorders. BP, Bipolar, MDD, Major depressive disorder, SCZ, schizophrenia.

In the table 5, we summarized potential biomarkers in gene association studies.

Gene	Methods	Results	Reference
Serotonin transporter	Association analysis	The low-expression variant (short allele) modifies risk of PTSD.	115-119
Cytomegalovirus gene (CMV)	Methylation microarrays to assay CpG sites from more than 14,000 genes, among 23 PTSD-affected and 77 PTSD-unaffected individuals.	CMV-a typically latent herpes virus whose activity was significantly higher among those with PTSD.	120
FK506 binding protein 5 (FKBP5)	1143 European Americans (EAs) and 1284 African Americans (AAs), screened for lifetime PTSD. 4 SNPs in FKBP5, rs3800373, rs9296158, rs1360780, and rs9470080, were genotyped.	In AAs, one of the SNPs, rs9470080, moderated the risk of PTSD that was associated with childhood abuse	121

Catechol-o-methyltransferase (COMT) (Val158Met)	Association analysis in 424 survivors of the Rwandan Genocide.	Met/Met homozygotes exhibited a high risk for PTSD independently of the severity of traumatic load.	122
Dopamine beta-hydroxylase (DBH) (1021C/T)	Association analysis in combat veterans with (N = 133) or without (N = 34) chronic PTSD.	A significantly lower plasma DBH activity in combat veterans with PTSD carrying the CC genotype	123
Interleukin-2 (IL-2) Interleukin-8 (IL-8)	34 earthquake survivors with PTSD (according to DSM-IV criteria), 30 earthquake survivors with non-PTSD and 34 controls in northern China	Earthquake survivors with PTSD had significantly lower serum IL-8 levels. PTSD may be associated with a reduced level of serum IL-8, and traumatic survivors may be associated with a lower level of serum IL-2.	80
C-reactive protein	3049 adults living in the community (Germany). CRP, lipoproteins and triglycerides determined. Also examined blood pressure, body mass index (BMI), physical activity, comorbid somatic diseases, medication, daily alcohol intake, and depression.	PTSD positive participants significantly higher odds for elevated CRP values than those without PTSD (OR=2.27; 95% CI: 1.32-3.93). Even after adjusting for sex, age, other sociodemographic factors, BMI, blood pressure, lipoproteins and triglycerides, physical activity, comorbid somatic diseases, daily alcohol intake, and trauma exposure, almost two-fold higher odds for elevated CRP levels in participants with PTSD.	124
NPY	qPCR, SNP, western blot	Low cerebrospinal fluid NPY plasma concentrations	125-127
P11	Real time PCR, PTSD post-mortem brain and blood cells	P11 over-expressed in the CNS and down-regulated in the blood	109, 128

Table 5. Association studies: genotype and symptoms - endophenotype

10. References

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6

Metabotropic Glutamate Receptors in Peripheral Tissues: Implications for Toxicology

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1. Introduction

Glutamate, the main excitatory neurotransmitter in the central nervous system (CNS), signals through ionotropic receptors (iGluRs), including AMPA, kainate and NMDA receptors, which are glutamate-gated ion channels and regulate rapid responses upon activation, and metabotropic receptors (mGluRs), which evoke slower responses through activation of intracellular transduction cascades. mGluRs are single peptide seven-transmembrane spanning proteins linked to intracellular G-proteins although it has been reported that G-protein-independent signalling can occur (Heuss et al., 1999). Eight different mGluRs (mGluR1–8) have been cloned and classified into three groups (groups I, II and III) based on sequence homology and the intracellular signal transduction pathways they activate. Group I metabotropic glutamate receptors include mGluR1 and mGluR5 subtypes, which activate phospholipase C and induce inositol triphosphate production and intracellular calcium mobilization. Group II mGluRs include mGluR2 and mGluR3 subtypes, whereas Group III mGluRs include mGluR4, mGluR6, mGluR7 and mGluR8 subtypes. All these receptors are negatively coupled to adenylyl cyclase signalling, resulting in inhibition of cyclic AMP production. Since mGluRs are expressed by neurons and glia near the synaptic cleft, where they modulate not only the effect of glutamate on the postsynaptic neurons but also the release of glutamate and other neurotransmitters, it is though that the mGluR system has evolved as a modulating mechanism for controlling excitability into the CNS (Schoepp, 2001). Furthermore, several mGluR subtypes were shown to exert glial and neuro-protective actions in distinct pathological conditions (Bruno et al., 1998; Kingston et al., 1999; D'Onofrio et al., 2001; Ciccarelli et al., 2007; Durand et al., 2010). However, mGluRs have currently received much attention motivated by a strong belief in their potential as drug targets for treatment of anxiety disorders and schizophrenia (Lavreysen & Dautzenberg, 2008; Chaki et al., 2010; Mezler et al., 2010; Schlumberger et al., 2009; Moreno et al., 2009; Patil et al., 2007).

The strongest suggestion that mGluRs are not exclusively synaptic receptors derives from numerous studies that demonstrate the presence of functional mGluRs in a number of peripheral non-neuronal cells, many of which do not even originate from the neural crest (Nicoletti et al., 2007), shifting the role of these receptors from mere synaptic regulators to modulators of basic cell functions (such as cell proliferation, differentiation and survival)

and key mediators of peripheral tissue function and neuroendocrine events. Besides organs that receive direct glutamatergic innervations, such as the heart and the adrenal glands, peripheral mGluRs can be activated in the absence of synaptic glutamate because of the existence of a large metabolic glutamate pool into cells derived from the Krebs cycle (Nicoletti et al., 2007). Then, metabolic glutamate can be transported outside the cell where it activates paracrine or autocrine mechanisms on cells expressing glutamate receptors.
As implied by the evidence above, the use of selective mGluR agonists and antagonists as therapeutic agents in treatment of anxiety disorders rises the problem of undesirable side effects in these patients. Therefore, in an effort to warn against unsafeness of clinical trials in the area of the anxiety disorders, this chapter summarizes current knowledge of the distribution and actions of mGluRs outside the brain.

2. mGluRs and anxiety

Several mGluR ligands were proved to have high efficacy for treatment of anxiety disorders: the mGluR1 antagonist JNJ16259685 and the mGluR5 antagonist MPEP showed anxiolytic effects in rodents (Spooren et al., 2000; Steckler et al., 2005a); group II mGluR agonists LY354740, LY379268 and LY404039 showed therapeutic actions in both anxiety and schizophrenia (Schoepp, 2004), whereas LY2140023 is entering phase II/III trials for schizophrenia (Eli Lilly, 2010).
While benzodiazepines exert their anxiolytic effect by binding to the γ-aminobutyric acid (GABA) receptors enhancing the inhibitory action of GABA in the CNS (Stahl, 2000); buspirone acts as a serotonin $(5\text{-HT})_{1A}$ receptor agonist (Ninan et al., 1998); propranolol is a β-blocker which arrests the autonomic arousal experienced during stress and anxiety (Noyes, 1985); and hydroxyzine is an antihistamine (Choy, 2007), the mechanism of anxiolytic action of mGluR ligands has not been completely elucidated. Currently, it is believed that their anxiolytic effects correlate with suppression of enhanced glutamatergic excitation at brain synapses involved in fear/anxiety in animals and humans (Schoepp et al., 2003), an action which is related to the synapse modulating function of these receptors. In addition, mGluR2/3 agonists regulate dopamine release and 5-HT2A receptor activity, which have been presumed to be involved in their antipsychotic action (Chaki et al., 2010).
It is now known that typical antipsychotic administration derives in multiple side effects such as extrapyramidal symptoms, tardive dyskinesia, hypertension or weight gain. Benzodiazepines, for example, although effective and well tolerated, present associated risks including drug-drug interactions, pregnancy problems, psychomotor impairment, memory problems, and physiologic dependence (Choy, 2007). More recently developed atypical antipsychotics like risperidone or clozapine also were associated with weight gain, diabetes, hyperlipidemia, arrhythmia, hyperprolactinemia-related sexual dysfunctions, dystonic reactions, caratact, and insomnia (Üçok & Gaebel, 2008). Therefore, it is noteworthy that mGluR ligands with antipsychotic properties were not shown to induce the commonest adverse effects at date. However, mGluR agonists/antagonists may present a different set of adverse effects, considering their vast range of target peripheral organs.

3. mGluRs in endocrine organs

The initial demonstration of a role for glutamate on neuroendocrine regulation resulted from the observation that neonatal administration of monosodium glutamate was followed

by brain lesions and obesity (Olney, 1969). Later, glutamate was shown to act on endocrine function mostly by modulating hypothalamic activity which in turn alters hormone release, but can also activate its receptors on pinealocytes, pancreatic cells, adrenal and sexual glands, further regulating hormone production and endocrine homeostasis.
Both glutamate and its receptors are localized in a variety of hypothalamic nuclei considered critical for reproduction and neuroendocrine function. mGluRs have been found not only in different regions within the hypothalamus such as the paraventricular nucleus (PVN), the ventromedial (VMN), arcuate (ARC), supraoptic (SON) nuclei and the preoptic area (POA), but also in the three lobes of the pituitary gland (Meeker et al., 1994), exerting regulatory actions on the hypothalamic-pituitary axis.

3.1 Effects of mGluRs on prolactin release

Glutamate regulates basal prolactin secretion and also affects the physiological response of this hormone to stimuli such as suckling and stress (Nagy et al., 2005). Johnson & Chamberlain (2002) suggested that LY379268, a group II mGluR agonist, produces disinhibition of tubero-infundibular dopamine release, which in turn increases prolactin secretion.
On the other hand, we demonstrated that group II mGluRs are present in lactotropes in rat anterior pituitary, which tallies with functional data showing that L-CCG-I, a group II mGluR agonist, decreases prolactin release from anterior pituitary gland (Caruso et al., 2004). We also showed that L-CCG-I induces apoptosis in lactotropes (Caruso et al., 2004), which may account for the inhibition in prolactin secretion exerted by these receptors.
Interestingly, we also observed that group II mGluR agonists LY379268 and LY354740 may have a partial dopamine agonist action since they bind to D2 receptors in rat striatum and to human cloned D2Long receptors in CHO cells (Seeman et al., 2008). This action could lead to an inhibition of prolactin release from anterior pituitary cells. Concordantly, LY379268 can reduce hyperprolactinemia under several conditions in rats (Johnson & Chamberlain, 2002). It is likely that the actions of these agonists *in vivo* involve both glutamate and dopamine components (Cartmell et al., 2000).
All these facts agree with the lack of hyperprolactinemia, one of the most frequent side effects of antipsychotic drugs, after mGluR ligand administration (Patil et al., 2007).

3.2 Effects of mGluR activation on hypothalamic neuropeptide release which regulates LH secretion

Evidence from our laboratory indicates that glutamate-induced release of substance P (SP) in the rat ARC and median eminence is mediated via activation of NMDA and group I mGlu receptors (Caruso et al., 2006). Since SP has been shown to stimulate prolactin and luteinizing hormone (LH) secretion, induction of SP release may mediate, at least in part, the stimulatory effect of glutamate on LH and prolactin secretion (Debeljuk & Lasaga, 1999).
Glutamate can regulate hypothalamic oxytocin release through activation of AMPA and mGlu receptors (Pampillo et al., 2001). Schrader & Tasker (1997a) found that activation of group I mGluRs reduced K^+ currents in SON magnocellular neurons, suggesting the presence of group I mGluRs in this hypothalamic area. In fact, we reported that group I mGluRs participate in the stimulatory effect of glutamate on hypothalamic oxytocin release in adult male rats (Pampillo et al., 2001). Morsette et al. (2001) also showed that group I mGluR activation increased oxytocin release in rat hypothalamo-neurohypophysial

explants. The increase in hypothalamic oxytocin release following group I mGluR activation would determine a rise in gonadotropin-releasing hormone (GnRH) release (van den Pol et al., 1990), and consequently a stimulation of LH release from the anterior pituitary. Interaction between oxytocin receptor and mGluR1 was proposed. In hypothalamic rat astrocytes, antagonism of mGluR1a leads to a decrease in oxytocin-induced $[Ca^{2+}]_i$ response whereas the agonist DHPG potentiates the oxytocin response (Kuo et al., 2009).
Morsette et al. (2001) demonstrated a concentration-dependent stimulation of vasopressin (VP) release when hypothalamo-neurohypophysial explants were perifused with group I mGluR agonists.
Activation of both group I and II mGluRs decreases alpha melanocyte stimulating hormone (α-MSH) release from hypothalamic fragments (Pampillo et al., 2002a). Since α-MSH inhibits preovulatory prolactin and LH surge and ovulation of female rats (Crown et al., 2007), mGluRs activity at α-MSH level could lead to increased LH production.
It is widely accepted that glutamate plays important roles in controlling GnRH neuron excitability, probably acting at the preoptic region of the hypothalamus (Gu et al., 1999). Only small sub-populations of GnRH neurons have functional mGluRs (Iremonger et al., 2010). A subpopulation of GnRH neurons in the medial septum was found to be excited by group I mGluR agonists (Dumalska et al., 2008). Nevertheless, we also know that activation of presynaptic group II/III mGluRs inhibits GABAergic input to GnRH neurons. Since GABA is involved in generation and modulation of the rhythm of GnRH release, mGluR could be affecting GnRH release (Chu & Moenter, 2005). Therefore, activation of mGluRs could inhibit GnRH release, at least in part thereby acting on GABAergic transmission. On the contrary, Lopez et al. (1992) and Pampillo et al. (2002b) reported that group I and II mGluR activation did not affect hypothalamic GnRH release *in vitro*.
In summary, actions of group I mGluRs on SP, oxytocin, VP and α-MSH at hypothalamic level seem to indicate that the use of group I mGluR antagonists as anxiolytic drugs could ultimately lead to decreased prolactin and LH surge.

3.3 Effect of mGluRs on Growth Hormone (GH) release

Aguilar et al. (2005) showed changes in GH secretion following administration of mGluR agonists to prepubertal animals: a significant decrease in serum GH concentrations after central (i.c.v.) administration of t-ACPD (a group I and II mGluR agonist) and following systemic administration of ibotenic acid (a weak agonist of all mGluRs). We have shown the presence of group II mGluRs in somatotropes and we observed an apoptotic effect of LCCG-I, a group II mGluR agonist, on this cell type (Caruso et al., 2004). mGluR inhibitory effects contrast with the potent stimulatory actions observed following iGluR activation (Aguilar et al., 2005). Thus, it becomes apparent that L-glutamate is able to exert a dual regulatory action upon GH secretion, which involves a predominant stimulatory effect via iGluRs, as well as a minor inhibitory effect via mGluRs.

3.4 Actions of mGluR activation on hypothalamic–pituitary–adrenal (HPA) axis

HPA axis is the key regulator of stress reaction. t-ACPD induced a significant increase in plasma corticosterone following i.c.v. administration (Lang & Ajmal, 1995). Jonhson et al. (2001) reported that treatment with either an agonist or antagonist of group I mGluRs results in a rise in serum corticosterone. The authors suggest that this paradoxical action may be due to a direct stimulatory effect of group I mGluR agonists on CRH release whereas selective

mGluR1 and mGluR5 antagonists may increase CRH release through disinhibiton of GABAergic interneurons. Concordantly, a selective mGluR5 antagonist increases circulating ACTH and corticosterone concentrations (Bradbury et al., 2003). On the other hand, an antagonist of group II mGluRs increased plasma corticosterone and CRH secretion from isolated hypothalami while group II mGluR agonists induced no modifications (Scaccianoce et al., 2003). The lack of effect of group II mGluR agonists supports the hypothesis that endogenous activation of group II mGluR could tonically inhibit hypothalamic CRH release.
It has also been demonstrated that i.c.v. administration of nonselective group III mGluR agonists L-AP4 and L-SOP induced an increase in corticosterone levels (Johnson et al., 2001). Mitsukawa et al. (2006) showed that mGluR7 subtype plays a role in the increase of stress hormones induced by group III mGluR agonists. AMN082, an allosteric agonist of mGluR7, induced a robust increase in stress hormone levels that was absent in mGluR7 knockout animals (Conn & Niswender, 2006). Group III mGluRs regulate the activity of GABA interneurones in the hypothalamus (Schrader & Tasker, 1997b) by decreasing L-glutamate release. Consequently, there would be decreased tone in GABAergic interneurons and a disinhibition of CRH neurons.
In summary, although the HPA axis is activated by mGluR1/5 antagonists and group III mGluR agonists, this effect does not seem to interfere with the anxiolytic role of these ligands.

3.5 Pancreas

Although the presence and function of iGluRs in pancreatic tissue is quite well defined, there is still no consensus regarding expression of mGluRs in pancreatic islets. Brice et al. (2002) found mGluR3 and mGluR5 mRNA and protein in rat and human islets of Langerhans; mGluR8 expression was detected in rat islets; and mGluR4 was detected in rat islets but not in α or β cell lines (therefore, they could be expressed in δ cells). In consonance with these findings, mGluR3, mGluR5 and mGluR8 activation improved release of insulin from a β cell line in the presence of glucose, although mGluR8 activation inhibited insulin release at higher glucose concentrations (Brice et al., 2002).
On the opposite side, Uehara et al. (2004) reported evidence for functional occurrence of mGluR4, but not other mGluRs, in alpha and F pancreatic cells, its activation showing an inhibitory effect on glucagon secretion by reducing cAMP production. Thus, mGluR4-mediated signaling pathway might provide a molecular basis for chemotherapeutics for hyperglycemia, one of the symptoms of type 2 diabetes. However, Tong et al. (2002) demonstrated mGluR8 (not mGluR4)-dependent inhibition of glucagon release from rat pancreatic islets. Taking a third position, Cabrera et al. (2008) found no effect of mGluR agonists on glucagon secretion using human islets.
In an animal model of diabetes, upregulation of mGluR5 causes cell damage and neurodegeneration (Anu et al., 2010). However, other authors reported that endogenous activation of mGluR5 is required for optimal insulin response to glucose in mice and is also involved in the correct glucagon response to insulin challenge (Storto et al., 2006). Intracerebroventricular injection of ACPD (a group I and II mGluR agonist) increases plasma glucose, insulin and glucagon levels (Lang & Ajmal, 1995). Adult mice lacking mGluR5 weighed significantly less than littermate controls and, on a high fat diet, mGluR5 -/- mice weighed less and had decreased plasma insulin and leptin concentrations (Bradbury et al., 2005).

3.6 Pineal gland

Pinealocytes express mGluR3 and mGluR5. Indeed, group II mGluR agonists inhibit norepinephrine-stimulated melatonin synthesis and N-acetyltransferase activity, possibly involving the mGluR3 subtype expressed in rat pineal gland (Yamada et al., 1998). Glutamatergic communication system of the pineal gland may not only enable paracrine crosstalk among pinealocytes but also probably relies on interactions between pinealocytes and interstitial cells analogous to neuronal-glial signaling (Pabst & Redecker, 1999). The evidence indicates that sleep disrupts can be associated with mGluR function.

4. Other organs

4.1 Kidney

In the rat kidney the presence of mGluR2/3 has been described in the juxtaglomerular apparatus and proximal tubules, suggesting that these receptors may be involved in electrolytes and water homeostasis (Gill & Pulido, 2001). In addition, strong immunoreactivity for mGluR2/3 was observed in granular cells of the afferent arteriole (Gill & Pulido, 2001), indicating a possible role in the control of renin release, a hormone which belongs to the renin-angiotensin system involved in regulation of electrolyte, fluid balance and blood pressure (Jackson et al., 1985). In human kidney, focal expression of mGluR4 was detected in the collecting duct (Chang et al., 2005), whereas positivity of a normal mouse glomerulus for mGluR7 was found along the glomerular basement membrane (Rastaldi et al., 2006).

4.2 Liver and gastrointestinal tract

One of the first studies reporting the presence of mGluRs in peripheral organs showed the ability of group I mGluR agonists to stimulate phosphoinositide hydrolysis in primary cultures of rat hepatocytes (Sureda et al., 1997). In subsequent studies, expression of mGluR5, but not mGluR1, and mGluR3 in rat liver was demonstrated (Do et al., 2007; Storto et al., 2000a). Moreover, mGluR3 were shown to be up-regulated in response to persistent hypoxic status such as fibrotic/cirrhotic conditions in rat liver macrophages, exerting a role in functional metabolism and viability in this tissue (Do et al., 2007), although it has been shown that an agonist of mGluR2/3 had no effect on rat hepatocyte death induced by anoxia (Storto et al., 2000a). On the contrary, endogenous mGluR5 activation is associated with liver damage induced by lipopolysaccharide and d-galactosamine (Jesse et al., 2009) or by acetaminophen in mice (Storto et al., 2003). In turn, selective blockade of mGluR5 protects against hepatocyte death induced by hypoxia (Storto et al., 2004) and oxidative stress (Storto et al., 2003) in rodents.

mGluR5 antagonists have proved to be useful in the treatment of gastroesophageal gastric reflux in clinical trials (Bolea et al., 2004; Zerbib et al., 2010). mGluR8 agonists also have protective effects on esophageal sphincter relaxation (Frisby et al., 2005). mGluR1-8 mRNA expression has been detected in different cell components of rat stomach mucosa (Nakamura et al., 2010), whereas intense mGluR2/3 protein staining was found in both parietal and endocrine cells, suggesting a role in the regulation of gastric acid and gastrin secretion (Gill & Pulido, 2001). mGluR1 is also located in glandular stomach and glutamate induces changes in the expression of pepsinogen (San Gabriel et al., 2007). mGluR1/5 signaling may increase intracellular pH in the duodenum (Akiba et al., 2009). The presence of mGluR2/3 and mGluR1/5 has been demonstrated in neurons of jejunum and ileum, suggesting that they may

play a role in the regulation of intestinal motility (Larzabal et al., 1999; Liu & Kirchgessner, 2000; Nasser et al., 2007). On the other hand, mGluR4 and mGluR7 are expressed in colon mucosa (Chang et al., 2005; Julio-Peper et al., 2010). Activation of mGluR7 in the colon could be a component of secretory disorders such as stress-induced diarrhea (Julio-Peper et al., 2010). mGluR8 is also present in the enteric nervous system and their activation by selective agonists increases colon motility (Tong & Kirchgessner, 2003).
Thus, apparently, no major adverse effects are expected to be induced by group I mGluR antagonists or group II mGluR agonists in the normal or anoxic/cirrhotic liver or in the gastrointestinal tract.

4.3 Reproductive system

Most currently available studies involving mGluRs in both female and male reproductive systems include only descriptive, anatomical analyses, although the unique distribution of mGluRs in sex organs suggests their participation in reproductive events such as germinal cell development, testicular development, sex hormone production and cyclic cell turnover.
In humans, immunoreactivity for mGluR1 was restricted to Leydig cells of intertubular spaces, where their activation could likely stimulate testosterone synthesis. mGluR5 was highly expressed in human seminiferous tubuli and in the mid-piece and tail of mature spermatozoa, even though neither mGluR5 agonist nor antagonist changed human sperm motility (Storto et al., 2001).
A strong immunolabeling for mGluR2/3 is present in the oocyte, the theca, and granulose cells in the macaque ovary (Gill et al., 2008). Likewise, in rat ovary, the oocyte showed intense staining for mGluR2/3, whereas the corpus luteum was moderately immunoreactive (Gill & Pulido, 2001). mGluR4 has been found in the human cervix and is weakly expressed in the endometrial glands (Chang et al., 2005). mGluR2/3 show positive immunoreactivity for the most superficial layer of the stratified squamous epithelium of the exocervix in rat uterus (Gill & Pulido, 2001). mGluR2/3 expression is predominant in proliferating ovarian and uterine structures, which indicates that its production may be cyclically regulated (Julio-Pieper et al., 2011).
All this evidence indicates that these receptors may be involved in ovulation, fertilization, implantation of the ovum and excitability of the uterus (Gill & Pulido, 2001). However, the only studies actually establishing a relationship between mGluRs and reproductive events are those which demonstrated a physiological interaction between estrogen receptors and mGluRs in both neurons and astrocytes (Dewing et al., 2007; Kuo et al., 2009). In the brain, estrogen receptor α interacts with mGluR1 to increase $[Ca^{2+}]_i$ flux and to initiate lordosis behavior and increases neuroprogesterone synthesis, which is a necessary step for estrogen positive feedback (Micevych et al., 2010). On the other hand, females treated neonatally with kainate, the type I/II metabotropic agonist ACPD, or both agonists combined showed adult male sexual behavior, indicating the participation of these glutamate receptor subtypes in masculinization (Wright & McCarthy, 2009).
Therefore, this evidence supports actual mGluR-mediated reproductive events and implications for fertility rise regarding administration of mGluR ligands for psychiatric disorders. Likewise, mGluRs are involved in embryonic development, as mGluR3 induce differentiation of neural stem cells (Ciceroni et al., 2010) whereas a switch from high mGluR5 expression to mGluR4 expression is found in embryoid bodies resembling embryogenesis (Cappuccio et al., 2006).

4.4 Immune system and thymus

mGluR activation has been proposed to play a similar role in the nervous and the immune systems by counteracting negative glutamate effects (Boldyrev et al., 2005). Since high levels of glutamate inhibit the proliferation of T-cells, glutamate has been related to immune deficiency (Ferrarese et al., 2001). Most attention has been focused on mGluR expression in thymocytes and T lymphocytes. Thymocytes express group I and II mGluRs (Storto et al., 2000b). Another study showed the presence of group III mGluRs in thymic cells (Rezzani et al., 2003). Group III mGluR activation may lead to oxidative stress and cell death of peripheral lymphocytes, a deleterious action potentiated by the presence of NMDA (Boldyrev et al., 2004, 2005). On the other hand, glutamate, acting via mGluR1 and mGluR5, has beneficial effects on human peripheral lymphocytes against activation-induced cell death (Miglio et al., 2005) or by inhibiting apoptosis induced by anti-CD3 treatment (Chiocchetti et al., 2006).

Pacheco et al. (2004) demonstrated that mGluR1 expression in human peripheral lymphocytes is detected after activation of the T-cell receptor CD3 complex, whereas mGluR5 is constitutively present (Pacheco et al., 2007), indicating that the expression of mGluRs in these immune cells depends on T-cell activation.

An antagonist of mGluR5 increased IL-6 secretion whereas an mGluR1 antagonist decreased the release of IL6, among other pro-inflammatory cytokines such as TNF-alpha and IFN-gamma (Pacheco et al., 2006), suggesting different signaling pathways of these mGluR subtypes in lymphocytes. Moreover, glutamate via group I mGluRs, regulates the initiation of T-cell-mediated immune responses (Pacheco et al., 2006).

mGluRs also seem to play a role in the development of autoimmune-related disorders (Julio-Pieper et al., 2011). For example, activation of mGluR4 in dendritic cells might exert a protective effect by preventing unbalance in T helper cells in a model of multiple sclerosis (Fallarino et al., 2010).

4.5 Heart

In animal and human hearts mGluR expression and effects on cardiac function have been reported. In rat heart, mGluR1, mGluR2/3 and mGluR5 are localized preferentially in the atrial nerve terminals, ganglion cells, and elements of the conducting system (Gill et al., 1999). In mouse heart, Moore-Morris et al. (2009) identified the mGluR1b transcript, which is functional in ventricular cardiomyocytes. In macaque heart, mGluR2/3 and 5 were found in myocardial nerve fibers, atrial intramural ganglia and myocytes, ventricular and submyocardial myocytes, Purkinje fibers, and bundle of Hiss (Mueller et al., 2003). In the human heart, mGluR1 and 5 but not mGluR2/3 were found in atrial intramural ganglia, atrial and ventricular cardiocytes, and bundle of Hiss (Gill et al., 2007).

Regarding the participation of the mGluR system in heart function, it has been shown that anteroventral third ventricular region infusion of mGluR agonist t-ACPD produced dose-dependent rises in plasma vasopressin, arterial pressure and heart rate after 5 or 15 min, although t-ACPD administration into the cerebral ventricle had no effect on these variables (Yamaguchi & Watanabe, 2004). Accordingly, group I, II and III mGluR agonists produced significant increases in arterial pressure and heart rate, although the respective antagonists failed to inhibit these cardiovascular responses (Tsuchihashi et al., 2000).

Nevertheless, opposite results were reported by others as follows. Microinjection of t-ACPD into the commissural subnucleus of the nucleus tractus solitarii elicited bradycardia (Braga et al., 2006). Activation of mGluR1, mGluR2/3, mGluR4 and mGluR8 into the nucleus tractus solitarius of anesthetized male Wistar rats elicited depressor and bradycardic

responses (Viard & Sapru, 2002). Furthermore, activation of spinal group I, II and III mGluRs increased the mean blood pressure in anesthetized rats while, after blockade of NMDA receptors, low doses of group II mGluR agonists induced hypotension and bradycardia (Celuch & García, 2002), suggesting that the main effects of mGluR agonist administration on cardiovascular function may depend on the dose used. Other authors have postulated that, in general, group I and II mGluRs produce responses consistent with excitation of neurons involved in reducing sympathetic outflow, heart rate, and arterial pressure (Foley et al., 1999; Jones et al., 1999).
These contradictory results make our interpretation of mGluR effects on cardiac function difficult. However, no adverse cardiovascular reactions were reported after mGluR ligand administration in patients with anxiety disorders.

4.6 Sense organs

mGluR1 and mGluR4, present in mammalian taste buds, sense umami taste elicited by monosodium glutamate (Chaudhari et al., 2009). The function of taste mGluR1 may be relevant in the back of the tongue. mGluR2 and mGluR3 mRNAs were also found in the circumvallate papillae, in cells co-expressing gustducin (Toyono et al., 2007).
mRNAs for seven of the eight mGluRs are expressed in the olfactory system, their expression being particularly high in the accessory olfactory bulb (Castro et al., 2007). In fact, under control conditions, recurrent inhibition of principal neurons (mitral cells) in accessory olfactory bulb slices was completely eliminated by mGluR1 antagonists (Castro et al., 2007). It has been suggested that mGluR2 might be involved in behavior associated with pheromone chemosignals (Nolte & Meredith, 2005).
All mGluRs except mGluR3 have been identified in the retina, with a differential distribution depending on the cell layers of retina (Connaughton, 2005), whereas mGluR6 activation have physiological significance (Gerber, 2003). In fact, defects in mGluR6 gene lead to congenital stationary blindness (Julio-Pieper et al., 2011). mGluR8 were clearly found in photoreceptor terminals in mammalian retina (Brandstätter et al., 1998; Koulen et al., 1999), their activation causing a decrease in $[Ca^{2+}]i$ in isolated rat photoreceptors (Koulen et al., 1999) and preventing glutamate excitotoxicity. mGluR1 and 5 may modulate responses of ON bipolar cells to neurotransmitters (Koulen et al., 1997). mGluR1/5, mGluR2 and mGluR4/7/8 have also been reported to be present in amacrine cells (Hartveit et al., 1995; Brandstätter et al., 1998) and rat ganglion cells (Akazawa et al., 1994; Hartveit et al., 1995).
Glutamate is thought to be the afferent neurotransmitter in the auditory system. In situ hybridization showed that mGluR1 alpha mRNA was expressed by type I and type II spiral ganglion neurons in the cochlea, although at low levels (Safieddine & Eybalin, 1995), suggesting that mGluR1alpha play a minor role in auditory transmission. Group I mGluRs expressed by SCC hair cells may serve as a mechanism for selective amplification of mechanically evoked transmitter release, thereby enhancing signal discrimination (Hendricson & Guth, 2002). Group I mGluRs contribute to neurotransmission between inner hair cells and afferent neurons in mammalian cochlea (Kleinlogel et al., 1999). Activation of group II mGluRs is able to increase the release of dopamine in guinea pig cochlea, via a disinhibitory mechanism involving local GABAergic fibers, reducing glutamate excitotoxicity (Doleviczényi et al., 2005). On the other hand, mGluR7 is expressed in hair cells and in spiral ganglion cells of the inner ear, being associated with age-related hearing impairment (Friedman et al., 2009).

The widespread distribution and multiple actions of mGluR subtypes in sense organs suggest possible deleterious effects of mGluR ligands on sense function.

4.7 Bone

Glutamate was identified in nerve fibers running through bone marrow in close contact with bone cells, suggesting that it may also act as a neuromediator in this tissue and may contribute to the regulation of bone remodeling (Chenu, 2002). Gu & Publicover (2000) reported expression of mGluR1 in rat femoral osteoblasts by RT-PCR, whereas only mGluR4 and 8 mRNAs were detected in rat cultured calvarial osteoblasts (Hinoi et al., 2001). The mRNA and protein for mGluR6 were identified in rat femoral marrow stromal cells from the osteoblast lineage, where their activation suppressed generation of nitric oxide, which is pivotal to bone physiology (Foreman et al., 2005). Occurrence of mGluR3, 5 and 8 mRNA was identified in mouse osteoclast, although only mGluR8 were found in mature osteoclasts (Morimoto et al., 2006). A specific mGluR8 agonist decreased KCl-evoked secretion of glutamate and bone degradation products (Morimoto et al., 2006). Therefore, glutamate, via mGluR8, is though to exert negative autocrine feedback, keeping osteoclasts in a suppressed state and preventing osteoporosis. In fact, vesicular glutamate transporter 1 (VGLUT1)-/- mice develop osteoporosis (Morimoto et al., 2006).
Cultured rat costal chondrocytes express mGluR1, 2, 4 and 8 and a group III mGluR agonist inhibits parathyroid hormone secretion through cAMP inhibition (Wang et al., 2005). Furthermore, chondral mineralization is greatly inhibited by group II and III mGluR activation in cultured embryonic mouse metatarsals by a mechanism involving apoptosis mediated by the depletion of intracellular glutathion (Wang et al., 2006).
Thus, there could be major implications for bone remodeling and chondral mineralization impairment by mGluR system-associated anxiolytic drugs.

5. mGluRs, tumor growth and cancer development

Stepulak et al. (2009) compiled data demonstrating that glutamate receptors are expressed in a variety of cancer cell lines (of neuronal and non-neuronal origin) and tumors, i.e., glioma, colorectal and gastric cancer, oral squamous cell carcinoma, prostate cancer, melanoma and osteosarcoma. It is also believed that the metabolic properties of tumors combined with altered metabolism in patients with cancer contribute to abnormally elevated glutamate plasma concentrations in these patients (Dröge et al., 1988). In turn, this excess of glutamate may activate its receptors and trigger intracellular signaling pathways, which may affect growth, survival and proliferation of cancer cells (Stepulak et al., 2009).
mGluR2 and mGluR7 were found to be expressed in all U87-MG and U343 (glioma), SK-NA-S (neuroblastoma), TE671 (rhabdomyosarcoma/medulloblastoma), MOGGCCM (astrocytoma), SK-LU-1 (lung carcinoma), HT29 and LS180 (colon adenocarcinoma), Jurkat E6.1 (T cell leukemia cells), RPMI 8226 (multiple myeloma), T47D (breast carcinoma), and FTC (thyroid carcinoma) cancer cell lines (Stepulak et al., 2009). Expression of the other 6 subtypes of mGluR varied between these cell lines, although they were present in most of them. Also, mGluR3 mRNA is increased by 5-fold in aldosterone-producing adenomas compared to normal human adrenal glands (Ye et al., 2007).
Ectopic expression of mGluR1 in human normal melanocytes, which normally lack this receptor, resulted in melanocyte hyperproliferation and transformation into malignant

tumors that set off distant metastases (Nicoletti et al., 2007; Marin & Chen, 2004). In the clinical setting, mGluR5 expression correlated with a decreased survival rate in patients with oral squamous cell carcinoma (Park et al., 2007) and an mGluR5 agonist increased tumor cell migration, invasion, and adhesion in human tongue cancer cells, an effect that was reversed by an mGluR5 antagonist (Park et al., 2007). On the contrary, in medulloblastoma, expression of mGluR4 was shown to be inversely related to tumor severity, spreading and recurrence (Iacovelli et al., 2006). Nevertheless, over-expression of mGluR4 was associated with poor prognosis in colorectal carcinoma (Chang et al., 2005) and their expression was identified in 68% of colorectal carcinomas, 50% of laryngeal carcinomas, and 46% of breast carcinomas (Julio-Pieper et al., 2011).
Concordant with the expression profile, pharmacological blockade of mGluR3 reduces cell proliferation and mitogen-activated protein kinase activation in cultured human glioma explants or glioma cell lines (D'Onofrio et al., 2003). Furthermore, systemic administration of the mGluR2/3 antagonist LY341495 inhibits the growth of glioma cells implanted either under the skin or inside the brain parenchyma of nude mice (Arcella et al., 2005). It is likely because of their neuroprotective role and the battery of trophic factors they induce that mGluR activation also stimulates glioma proliferation.

6. Concluding remarks

mGluRs play important neuromodulatory roles throughout the brain as such they are targets for therapeutic intervention for a number of psychiatric and neurological disorders including anxiety, depression, Parkinson's disease and schizophrenia.
Currently approved antipsychotic drugs have substantial extrapyramidal and metabolic side effects, but (beyond its high efficacy and ease of delivery) the advantage of use of mGluR ligands for the treatment of anxiety disorders is the lack of the commonest undesirable effects, which include alterations in prolactin levels, extrapyramidal symptoms, weight gain, glucose abnormalities, hypertension, sedation or Parkinsonian symptoms. The probability of a complete absence of adverse effects, however, does not seem to be very high because, as implied by the evidence compiled in this chapter, the implications of mGluR ligands toxicity are many and far-reaching.
mGluR1 selective antagonists showed efficacy in rodent models of anxiety, however, these compounds were associated with memory impairment that interrupted further development (Steckler et al., 2005a,b; Gravius et al., 2005). On the other hand, mGluR5 selective antagonists, which also has anxiolytic efficacy did not cause impairment in memory (Steckler et al., 2005a; Gravius et al., 2005).
As reported (Mezler et al 2010; Chaki et al 2010; Patil et al 2007), the latest developed allosteric group II mGluR agonists have efficacy in preclinical models of psychosis and anxiety, without involving any of the most frequent side effects associated with typical and atypical antipsychotics. Nevertheless, the ubiquity of mGluRs in peripheral tissues and the broad spectrum of possible side effects signify that the reported measurements are far from exhaustive. Consequently, some "silent" or long term unacceptable side effects on other target organs might be associated with mGluR agonist administration and should be considered. Endocrine alterations such as changes in LH, GH or oxytocin levels induced by group I mGluR ligands should be taken into consideration. Furthermore, fertility impairment and embryogenesis defects, immune deficits, sense function impairment and osteoporosis induced by group II mGluR activation are issues to be further studied. Finally,

tumor development after mGluR ligand administration represents another possible risk to be further investigated. Hence, there is a need for screening and monitoring for all these possible problems, which are not considered in current clinical trials.

7. References

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7

The Carioca High and Low Conditioned Freezing Lines: A New Animal Model of Generalized Anxiety Disorder

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1. Introduction

Fear and anxiety are complex concepts. Both terms have been used to describe a set of highly orchestrated neural events that involve sensory processing and motor responses triggered by threatening situations. These events are mediated by central neural circuitries and peripheral neuroendocrine pathways and clearly have adaptive value. Sensory systems function as alerting signals to warn of real or potential danger, producing a shift to a state of high vigilance that prepares the individual to avoid or escape from a wide variety of dangerous situations. Most of these reactions are not exclusive to our species. Because of their importance for survival, fear and anxiety traits are believed to have been selected in human evolution and shaped by natural selection for their crucial role in protecting individuals who face adverse environments (Coutinho et al., 2010; Gross & Hen, 2004; Marks & Nesse, 1994).

However, these highly adaptive events can be disabling when the individual experiences them excessively or when they occur in the absence of threatening stimuli. In these cases, they represent a pathological condition termed an anxiety disorder. Often chronic in nature, these disorders are among the most prevalent mental health problems across the individual life span, producing severe impairments in social and occupational functioning.

According to an evolutionary perspective, an anxiety disorder reflects a malfunctioning of the neural circuits responsible for detecting, organizing, or expressing adaptive defense reactions (Jacobson & Cryan, 2010). Humans and nonhuman mammals share approximately the same behavioral defense strategies, reflected by activation of similar underlying neural circuitry. Therefore, animal models of anxiety can be extremely helpful for better understanding the behavioral, neural, and genetic substrates involved in these pathologies. The purpose of the present chapter is to present two new lines of rats that might be a useful model of generalized anxiety disorder (GAD). Before we discuss this model, defining how anxiety disorders are currently classified is important.

2. Clinical aspects of anxiety

The concept of anxiety disorders has changed dramatically over the years as more clinical and experimental evidence has been collected. In the clinical setting, anxiety disorders

departed from a single construct that ranged in intensity from normal to pathological or neurotic levels. A major shift in this view occurred with Klein's pioneering work (Klein, 1964; Klein & Fink, 1962), which showed that imipramine had a selective effect in the treatment of panic disorder. Moreover, certain anxiety disorders have been suggested to differ from each other in the primary object or specificity of threat. Fear of a circumscribed and well-defined object is a characteristic of specific phobias, whereas diffuse and chronic sustained anxiety is the main feature of GAD.

The 3rd edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-III; American Psychiatric Association, 1980) introduced the current descriptive symptom-based approach to mental disorders with well-defined, explicit diagnostic criteria. This new classification incorporated distinct nosological entities, such as panic disorder, specific and social phobias, GAD, posttraumatic stress disorder, and obsessive-compulsive disorder. In the DSM-III, GAD was left as a residual diagnosis of worry, to be made only in the absence of other anxiety and depressive syndromes. Consequently, this residual category carried low diagnostic reliability.

With the publication of the DSM-IV (American Psychiatric Association, 1994) and *International Classification of Diseases and Related Health Problems* (ICD-10; World Health Organization, 1992), these anxiety disorder categories remained basically the same. However, the diagnosis of GAD shifted from a residual category in the DSM-III to an independent anxiety disorder type in the DSM-IV. Free-floating anxiety was associated with the worry construct, which in turn produced several symptoms, such as muscle tension, fatigue, restlessness, concentration difficulties, and irritability. According to the DSM-IV, excessive and unrelenting worry is generally associated with impairments in academic, social, and personal functioning and related to multiple domains or activities. To be considered a pathological feature of GAD, worry must occur more days than not for a period of at least 6 months.

3. Animal models of anxiety

In the experimental setting, most of the studies that investigate the etiological mechanisms that underlie anxiety disorders have been performed using animal models. Defensive reactions of the laboratory rat (*Rattus norvegicus*) have been employed as the main system for modeling human anxiety. Defecation in the open field was probably one of the first animal models of anxiety (Hall, 1934). Since then, several other animal models of anxiety have been developed. As in the clinical setting, the traditional view that highlighted these experimental studies was that animal defensive responses were mediated by a single and general anxiety construct (Broadhurst, 1975; Gray, 1979; Hall, 1934). Nevertheless, as new data were collected, it became clear that animal defensive behavior is mediated by a complex and multidimensional construct (Aguilar et al., 2002; Belzung & Le Pape, 1994; Ramos et al., 1997; Torrejais et al., 2008). In these studies, statistical techniques, such as factor analysis, were employed to investigate whether different animal models of anxiety measure the same underlying latent factor. The results clearly indicated that different animal models assessed distinct forms of anxiety. For example, File (1992) showed that indices of anxiety derived from the elevated plus maze (i.e., the number of entries into and time spent on the open arms of the maze), Vogel conflict test (i.e., frequency of punished drinking), and social interaction test (i.e., time spent engaged in social interaction), loaded on three independent factors, suggesting the existence of different forms of anxiety generated by each of these paradigms.

Pharmacological studies that employed diverse animal models also confirmed the multidimensional aspect of anxiety. For example, benzodiazepine compounds produced an anxiolytic effect in animal models that generate behavioral inhibition caused by the conflict between approach and avoidance tendencies (Maki et al., 2000). These animal models also indicated that substances that decrease serotonergic neurotransmission increase anxiety, whereas compounds that increase serotonergic neurotransmission decrease anxiety (Graeff, 1997). In contrast, other animal models that require vigorous escape responses to proximal aversive stimuli appear to be resistant to benzodiazepine drugs, whereas substances that increase serotonergic activity produce an anxiolytic effect (Graeff and Zangrossi, 2010).

Different neural circuitries appear to be involved in distinct dimensions of anxiety. Gray and McNaughton (2000) argued that the septo-hippocampal system contributes to the cognitive component (worry), and the amygdaloid complex and its projections to the ventral portion of the periaqueductal gray (PAG) are critically involved in the regulation of inhibitory behavior in response to innate or conditioned aversive stimuli (Fanselow, 1994). Active defensive behaviors in response to proximal stimuli, generally associated with nociception, appear to involve the dorsal portion of the PAG (dPAG) and its ascending projections to forebrain structures related to the sensorial processing of aversive stimuli (Oliveira et al., 2004).

These diverse dimensions found in animal models of anxiety may indicate that clinically defined anxiety disorders could be associated with a particular animal model. However, the adoption of descriptive and operational criteria from the modern classification systems imposed a validity problem among the several anxiety disorder categories. The DSM-IV and ICD-10 are not primarily based upon etiology, neurobiology, epidemiology, genetics, or responses to medications, but rather on phenomenological descriptions of clinical data that have imprecise similarity or correlate with each other within and between individuals (Gould & Gottesman, 2006). Therefore, unsurprising are the several problems that are encountered when attempting to use the current systems of mental disorder classification as a guide for developing viable animal models.

4. Contextual fear conditioning as a model of generalized anxiety disorder

Regardless of the difficulty developing animal models for current clinically defined anxiety disorders, fear conditioning has been historically associated with one of the main causes of pathological anxiety (i.e., neurosis; Pavlov, 1927; Watson & Rayner, 1920). In a typical fear conditioning experiment, a discrete and emotionally neutral stimulus, such as a light or tone, reliably signals the occurrence of an aversive stimulus, such as an electric footshock. After a few pairings between these two stimuli, the previously harmless stimulus becomes a potent conditioned stimulus (CS) and acquires the ability to elicit several fear reactions.

Another form of fear conditioning is to make the aversive stimulus unpredictable. According to this alternative procedure, a rat is exposed to a novel chamber and, after a few minutes, a brief and unsignaled footshock is delivered. When returned to the same chamber in the absence of the aversive stimulus, the animal presents a permanent fear reaction to contextual cues previously associated with the footshock.

Considerable evidence from animal and human experiments indicate that fear conditioning in response to a discrete CS and contextual cues is mediated by different neural circuitries (Indovina et al., 2011; Ferreira et al., 2003; Kim & Fanselow, 1992; LeDoux, 2000; Pohlack et al., 2011). These results support the hypothesis of at least two dimensions of fear conditioning, and

each dimension might be related to clinically distinct anxiety disorders. Specific phobias, characterized by cue-specific or phasic fear reactivity, might be modeled by aversive conditioning in response to a discrete CS (Grillon, 2002; Grillon and Davis, 1997). GAD, in contrast, is characterized by persistent and diffuse or non-cue-specific anxiety and might be modeled by contextual fear conditioning (Brandão et al., 2008; Grillon and Davis, 1997).

Contextual fear conditioning represents one of the simplest and most rapid forms of producing aversive learning (Landeira-Fernandez, 1996). Defensive freezing behavior has been argued to be the most reliable measure of contextual fear conditioning (Fanselow, 1984a). This defensive response is a direct function of shock intensity (Sigmundi et al., 1980) and depends on the association between the cues of the experimental chamber and footshock (Landeira-Fernandez et al., 2006).

Conditioned freezing in response to contextual cues previously associated with footshock has been pharmacologically validated as an adequate model of anxiety disorder. Accordingly, classic anxiolytic benzodiazepines, such as midazolam and diazepam (Fanselow and Helmstetter, 1988), and non-benzodiazepine anxiolytics, such as the serotonin-1A (5-hydroxytryptamine-1A [5-HT_{1A}]) receptor agonist ipsapirone (Inoue, Tsuchiya, Koyama, 1996) and 5-HT reuptake inhibitors citalopram and fluvoxamine (Hashimoto et al., 1996), reduced the amount of conditioned freezing. Furthermore, anxiogenic substances, such the benzodiazepine inverse agonist dimethoxy-β-carboline, produced freezing behavior similar to that elicited by fear conditioning (Fanselow et al., 1991).

5. The Carioca High and Low conditioned Freezing rats

Bidirectional selective breeding of a defensive response or any other phenotypic characteristic is a technique in which animals are bred to modify the frequency of the genes that underlie a particular phenotype. Mating animals within a population based on the opposite extremes of an observable characteristic will push, over many generations, this particular phenotype in opposite directions, leading to two separately bred lines. This technique has been widely employed to investigate how genes can influence various behavioral traits, including defensive reactions associated with emotionality. In particular, genetic animal models of anxiety disorders might be a useful tool for understanding why some individuals present adequate emotional reactions and others endure an exaggerated pattern of anxiety responses in the absence of a fear-provoking context.

The view that anxiety does not reflect a single or unitary process emphasizes the importance of developing different genetic models with distinct phenotype criteria. In fact, the development of bidirectional lines of animals with high and low levels of emotionality began in the middle of the 20th century. Since then, a relatively large number of different lines have been described in the literature (for review, see Ramos and Mormède, 2006). Innate and learned animal models have been employed for mating selection in rats. Among the innate models are defecation (Maudsley animals; Broadhurst, 1957, 1958) and ambulation in the center of an open field apparatus (Floripa animals; Ramos et al., 2003), ambulation on a runway (Tsukuba animals; Fujita, 1984), open arm parameters in the elevated plus maze (HAB and LAB animals; Liebsch et al., 1998a, b), and infant isolation-induced ultrasonic vocalizations (USV animals; Brunelli & Hofer, 1996). Surprisingly, the two-way-avoidance response has been the main conditioned phenotype criterion used for developing bidirectionally selected rat lines based on learned aversive paradigms. That is

the case for Roman (Bignami, 1965), Syracuse (Brush et al., 1979), Australian (Bammer, 1983), Koltushi (Ryzhova et al., 1983), and Hatano (Ohta et al., 1995) animals.

Our group in the Psychology Department at Pontifícia Universidade Católica do Rio de Janeiro (PUC-Rio) was also interested in developing a rat genetic model of extreme phenotypes of learned fear. Instead of the two-way avoidance paradigm, conditioned freezing in response to contextual cues previously associated with footshock was employed as the phenotype criterion for developing the two lines. The breeding program began in 2006. The basic protocol consisted of mating male and female albino Wistar rats with the highest and lowest conditioned freezing in response to the contextual cues of the experimental chamber where animals were exposed to three unsignaled electric footshocks on the previous day. Gomes and Landeira-Fernandez (2008) found that after three generations, reliable differences between these two lines were already present, indicating a strong heritable component of this type of learning. The lines were named Carioca[1] High conditioned Freezing (CHF) and Carioca Low conditioned Freezing (CLF). These two lines represent the most recent rat genetic model in the field of anxiety.

6. Phenotype results of the 12th generation

To illustrate the development of our breeding lines, we present the phenotype results of the 12th generation of the CHF and CLF lines recently collected in our laboratory. A random (RND) line of randomly selected rats was also used as a control group for the CHF and CLF lines. Phenotyping was performed on a total of 122 animals from the CHF line (67 males and 55 females), 124 animals from the RND line (54 males and 70 females), and 99 animals from the CLF line (49 males and 50 females).

Animals were born and maintained in the colony room of the PUC-Rio Psychology Department with controlled room temperature (24 ± 1°C) and a 12 h/12 h light/dark cycle (07:00-19:00 h). To assign a control number for each animal, amputation of one toe from each foot and a small incision in one of the ears was performed 6 to 8 days after birth. Upon weaning at 21 days of age, each animal was separated by sex and housed in groups of five to seven, according to their respective lines, in polycarbonate cages (18 × 31 × 38 cm) with food and water available *ad libitum*. Phenotyping occurred during the light phase of the cycle. The animals were between 75 and 80 days of age at the beginning of the experiment. For 5 days before the contextual fear conditioning experiment, the animals were handled once daily for a period of 2 min.

Contextual fear conditioning occurred in four observation chambers (25 × 20 × 20 cm), each placed inside a sound-attenuating box. A red light bulb (25 W) was placed inside the box, and a video camera was mounted in the back of the observation chamber so the animal's behavior could be observed on a monitor outside the experimental chamber. A ventilation fan attached to the box supplied background noise of 78 dB (A scale). The floor of the observation chamber consisted of 15 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center), which were wired to a shock generator and scrambler (Insight, São Paulo, Brazil). An interface with eight channels (Insight) connected the shock generator to a computer, which allowed the experimenter to apply an electric footshock. Ammonium hydroxide solution (5%) was used to clean the chamber before and after each subject.

[1] Carioca is the name given to those born in Rio de Janeiro.

The contextual fear conditioning protocol involved one acquisition session and one test session. During acquisition, each animal was placed in the observation chamber for 8 min. At the end of this period, three unsignaled 0.6 mA, 1 s electric footshocks were delivered with an intershock interval of 20 s. Three minutes after the last footshock (post-shock interval), the animal was returned to its home cage. The test session occurred approximately 24 h after training. This test consisted of placing the animal for 8 min in the same chamber in which the three footshocks were delivered on the previous day. No footshock or other stimulation occurred during this period. A time-sampling procedure was used to evaluate fear conditioning in response to contextual cues. Every 2 s, the animal was observed, and a well-trained observer recorded episodes of freezing, defined as the total absence of movement of the body or vibrissa, with the exception of movements required for respiration.

Previous results from our laboratory indicated that male rats consistently exhibited more conditioned freezing in response to contextual cues than female animals (Gomes and Landeira-Fernandez, 2008). Therefore, male and female results are presented separately. Fig. 1 presents the mean ± standard error of the mean (SEM) percentage of time spent freezing among male and female rats of the CHF, RND, and CLF lines during the post-shock period. The results were analyzed using a two-way analysis of variance (ANOVA). The first factor, with two levels, was related to the animal's sex (male and female). The second factor, with three levels, was related to the breeding line (CHF, RDN, and CLF).

This analysis revealed an absence of a two-way interaction ($F_{2,339}$ = 0.44, p > 0.6). A main effect of sex was found ($F_{1,339}$ = 14.02, p < 0.001). As shown in Fig. 1, male rats expressed more freezing behavior than female rats across all three levels of the breeding line factors. A main effect of breeding line was also detected ($F_{2,339}$ = 20.27, p < 0.001). Pairwise *post hoc* comparisons performed with Fisher's Least Significant Difference test indicated that CLF animals expressed lower freezing behavior compared with CHF and RND animals (all p < 0.001). Finally, CHF and RND animals did not differ significantly from each other (p > 0.4).

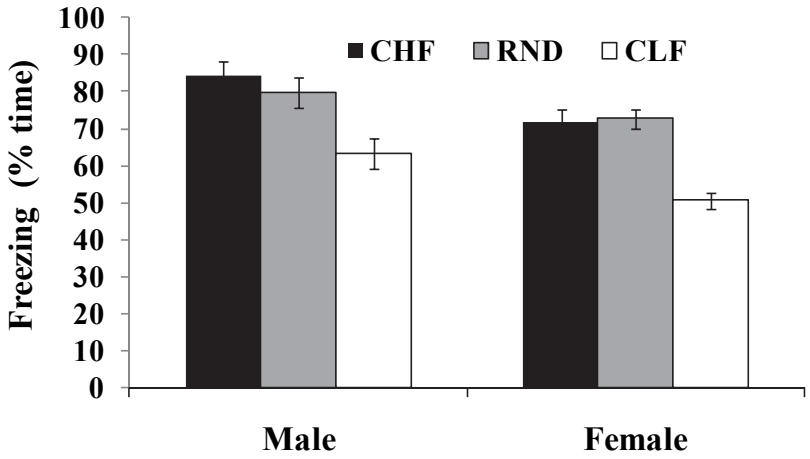

Fig. 1. Mean ± SEM percentage of time spent freezing among male and female rats of the high (CHF), random (RND), and low (CLF) lines during the post-shock period of the training session.

The differences observed in the amount of post-shock freezing behavior between CLF and CHL animals were not observed in our original report that employed the 3rd generation of these two lines (Gomes and Landeira-Fernandez, 2008). Three possibilities may explain these discrepant results. One is that the footshock intensity used to phenotype animals of the present generation (0.6 mA) was much lower than the intensity used during the first three generations (1.0 mA) reported by Gomes and Landeira-Fenandez (2008). Therefore, the higher footshock intensity could lead to a ceiling effect so that differences in post-shock freezing behavior may not be observed. Indeed, the footshock intensity was reduced in our breeding program in the 7th generation to 0.7 mA and in the 8th generation to the present intensity to prevent possible ceiling effects produced by this relatively strong (1.0 mA) footshock intensity.

A second possibility could be related to the fact that freezing observed immediately after footshock reflects associative learning between contextual cues and the aversive footshock (Fanselow, 1980, 1990; Vianna et al., 2001b). For example, when the footshock is presented simultaneously with the rat's placement in the chamber, no contextual fear conditioning is observed (Landeira-Fernandez et al., 1995). Moreover, placing the animal in a different context from the one in which the footshock was delivered did not produce any freezing behavior (Fanselow, 1980). Therefore, differences between CHF and CLF animals in post-shock freezing could be a consequence of the fact that CHF rats have a greater propensity for exhibiting higher conditioned freezing responses compared with CLF animals because of the continuous bidirectional selection over different generations.

A third possible explanation for these incongruent results might be related to differences in pain sensitivity between these two lines. This is an important issue because freezing observed immediately after footshock is closely related to pain sensitivity and shock intensity (Fanselow, 1984b). According to this possibility, selection for high and low conditioned freezing might independently lead to co-selection of other contributing factors that are not genetically linked but contribute to the phenotype that is being selected, such as differences in pain sensitivity to footshock. Further studies are necessary to test this possibility.

Fig. 2 presents the mean and SEM percentage of time spent freezing among male and female rats of the high (CHF), random (RND), and low (CLF) lines during the 8 min test session. Conditioned freezing in response to contextual cues previously associated with footshock was also analyzed using a two-way ANOVA. This analysis indicated an absence of a two-way interaction ($F_{2,339} = 0.07$, $p > 0.9$). A main effect of sex was found ($F_{1,339} = 41.85$, $p < 0.001$). As shown in Fig. 2, male rats froze more than female rats across all three levels of the breeding line factors. A main effect of breeding line was also detected ($F_{2,339} = 18.13$, $p < 0.001$). Fig. 2 also shows that the CHF line expressed the highest amount of conditioned freezing, and the CLF line expressed the lowest amount of freezing. The RND line presented intermediate levels of freezing. These results were confirmed by pairwise *post hoc* comparisons. CHL animals froze more than RND and CLF animals, and CLF rats froze less than CHF and RDN animals (all $p < 0.01$).

Electric footshock induced a reliable difference between CHF and CLF animals, and we evaluated whether the breeding line effect on conditioned freezing during the test session was attributable to post-shock differences that these animals presented during the training session. An analysis of covariance, with post-shock as a covariant factor, was performed. The results from this analysis confirmed an absence of an interaction ($F_{2,338} = 0.19$, $p > 0.8$) and main effects of sex ($F_{1,338} = 31.14$, $p < 0.001$) and breeding line ($F_{2,338} = 10.23$, $p < 0.001$).

These results confirmed previous findings from our original report (Gomes and Landeira-Fernandez, 2008) and extend these results to a control group of animals that were randomly mated.

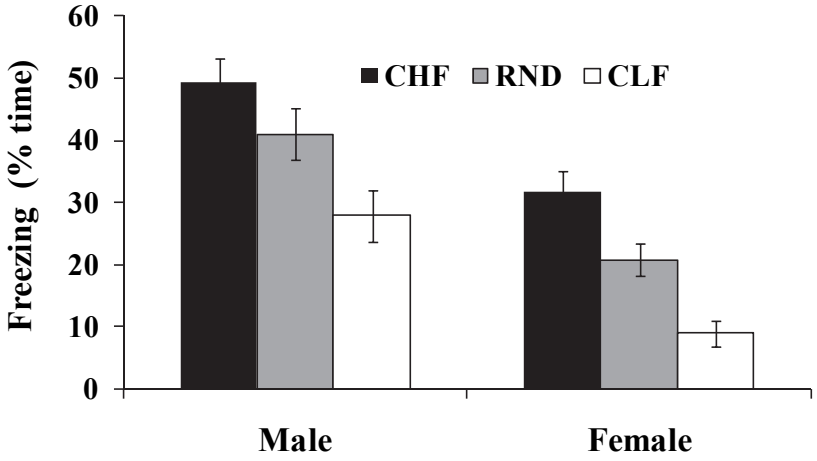

Fig. 2. Mean ± SEM percentage of time spent freezing among male and female rats of the high (CHF), random (RND), and low (CLF) lines during the test session 24 h after training.

7. Behavioral validation of the Carioca lines

An important issue in the processes of developing a new genetic animal model of anxiety is to evaluate whether the pair of contrasting lines of rats selectively bred for high and low anxiety-related responses also display convergent results in other threatening situations that also require the activation of defensive responses. The first behavioral results from this ongoing selective breeding program were reported by Dias et al. (2009). They performed a battery of behavioral tests that evaluated the emotional and cognitive aspects of the 4th generation of the CHF and RND lines. To evaluate anxiety-related behaviors, the CHF and RND lines were tested in the elevated plus maze and social interaction test. CHF animals were significantly more emotionally reactive than RDN rats in terms of both the number of entries into and time spent on the open arms of the elevated plus maze. The time spent engaged in social interaction behavior was also significantly decreased. Importantly, no differences were found in locomotor activity, measured by the number of entries into the closed arms of the elevated plus maze and number of crossings in the social interaction test arena. Therefore, motor activity did not account for the differences between CHF and RDN animals.

Dias et al. (2009) also found an absence of differences between the CHF and RND lines in the forced swim test, suggesting that the anxiety trait selected in the CHF line did not interact with affective disorder traits, such as those for depression. The cognitive aspects of CHF rats were evaluated in the object recognition task. The results from this test indicated no difference between the two groups. These negative results indicated that our breeding procedure, which increased the occurrence of conditioned freezing in response to contextual

cues previously associated with footshock, did not interfere with other emotional or memory systems. Although these results are extremely encouraging, additional experiments are necessary to further evaluate the behavioral profile of each of these lines.

8. Panic-related behaviours in the Carioca lines

Panic disorder is a complex anxiety disorder that involves both recurrent, unexpected panic attacks and persistent concern about having additional attacks (American Psychiatric Association, 1994). Although the occurrence of a panic attack is a hallmark of panic disorder, the chronic conditioning of this anxiety disorder is defined by the constant and persistent fear of experiencing further attacks or worry about the possible consequences of a panic attack.

The clinical concept of panic attack and panic disorder is well described in the literature (Freire et al., 2010). However, the relationship between an anticipatory anxiety trait present in GAD with panic attack and the development of panic disorder remains a subject of intense debate (Battaglia and Ogliari, 2005; Bouton et al., 2001; Stein et al., 2010). The distinction between panic disorder and GAD stemmed from Klein´s original observations (Klein, 1964; Klein and Fink, 1962), in which chronic administration of the antidepressant imipramine improved panic disorder, which was resistant to benzodiazepine anxiolytics at doses that improved GAD. This pharmacological distinction between these two anxiety disorder categories has been further qualified. Chronic imipramine also improves GAD (Kahn et al. 1986), and high-potency benzodiazepines, such as alprazolam, are effective in panic disorder when chronically administered (Schweizer et al. 1993).

Empirical research has successfully employed electrical stimulation of the dPAG as a useful animal model of both panic attack (i.e., the acute reaction that might trigger the panic disorder condition) and panic disorder (i.e., the chronic or continuous condition that characterizes the full expression of this anxiety disorder). A stepwise increase in the electrical current intensity used to stimulate the dPAG in rats produces a suppression of spontaneous locomotor activity (i.e., freezing) accompanied by piloerection and exophthalmus at lower intensities. As stimulation continues, active escape behaviors, such as running and jumping, appear at higher intensities (Brandão et at., 1982). After the termination of the dPAG electrical stimulation at the escape threshold, the animal engages in a long-lasting freezing response (Vianna et al., 2001a). Freezing and escape responses triggered by electrical stimulation of the dPAG represent a model of panic attack, whereas dPAG post-stimulation freezing at the aversive escape threshold appears to be a model of panic disorder (for review, see Brandão et al., 2008).

Recently, Galvão et al. (in press) exposed CHF and CLF animals from the 9th generation to the dPAG electrical stimulation paradigm. The results indicated that CHF animals had a higher dPAG electrical stimulation aversive threshold for producing freezing and escape reactions than CLF animals. However, CHF animals displayed more freezing behavior immediately after dPAG electrical stimulation at the escape threshold compared with CLF animals. Thus, although CHF animals were more resistant to the expression of freezing and escape behavior in response to dPAG stimulation, they were more prone to freezing after the occurrence of the dPAG aversive stimulation compared with CLF animals. These results are consistent with the interpretation that although anticipatory anxiety might exert an inhibitory effect on the expression of panic attack, it might also facilitate the pathogenesis of panic disorder.

9. Conclusions

Anxiety disorders are among the most prevalent mental health problems across the individual life span. Early clinical and experimental conceptualizations of anxiety departed from a single or unitary general trait model. More recent theories have favored the view that anxiety is a complex, multidimensional, and dynamic phenomenon. Animal modeling has been crucial in dissecting the pathophysiological mechanisms and designing more effective therapies. Contextual fear conditioning has clear isomorphism with GAD, whereas electrical stimulation of the dPAG appears to be a valid animal model of panic attack and panic disorders.

Bidirectional selection for high and low anxiety-like behavior is a valuable tool for understanding the neural substrates of anxiety disorders. Our laboratory recently developed two new lines of Wistar rats, CHF and CLF, that were selectively bred for high and low levels of freezing in response to contextual cues previously associated with footshock. After three generations of breeding, CHF rats were considered to have a greater propensity for exhibiting higher conditioned freezing responses compared with CLF animals. The present phenotype results of our 12th generation indicated that CHF and CLF lines differed from each other and from a RND control line. CHF and CLF animals also presented a difference in freezing triggered immediately after the occurrence of footshock.

The results from the 4th generation also indicated that CHF animals were more "anxious" than RND rats in the elevated plus maze and social interaction test. Motor activity did not account for the differences between the CHF and RND lines. The absence of reliable differences between CHF and RND animals in the forced swim test and object recognition task indicated that the breeding procedure, which increased the occurrence of conditioned freezing in response to contextual cues, did not interfere with other emotional or memory systems. Finally, exposure of CHL and CLF animals to electrical stimulation of the dPAG suggested that the component of anticipatory anxiety present in GAD might exert an inhibitory effect on the expression of panic attack, whereas it might also facilitate the pathogenesis of panic disorder.

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8

The Loss of Glutamate-GABA Harmony in Anxiety Disorders

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1. Introduction

The proper functioning of the central nervous system (CNS) depends on the physiological homeostasis, which is itself maintained and regulated by two opposite forces acting independently to each other, flowing into a natural cycle and always seeking the balance. The thing is about two main amino acid neurotransmitters, glutamate and GABA, creating the opposite excitatory/inhibitory forces in the brain. Together, these two neurotransmitters constitute more than 90% of all neurotransmission, leaving less than 10% for the others. Therefore, to all the possibilities their mutual interaction determinates the proper functioning of the CNS. In Fig. 1, the schematic balance is presented, which mirrors the physiological equilibrium between GABA (represented by the white dots) and glutamate (represented as the black dots).

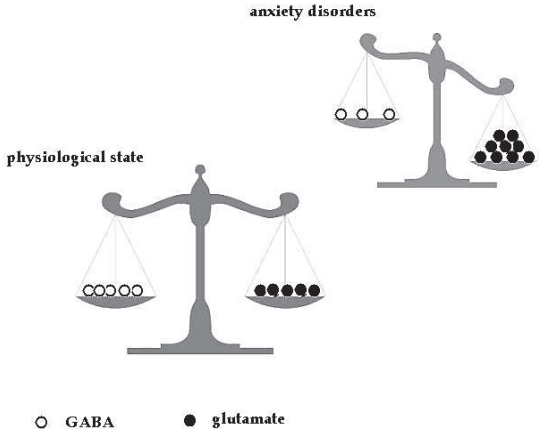

Fig. 1. The schematic balance presenting the equilibrium between GABA and glutamate in the physiological state and its loss (overactivation of glutamatergic system) in the anxiety disorders.

A variety of mechanisms keep the inhibitory/excitatory forces on the physiological level in the CNS. The disruption of the cycle leads, in consequence, to the advantage of one amino acid over another, resulting in psychiatric disorders. In the anxiety disorders that inhibitory/excitatory equilibrium is twisted into increased glutamate level, which will be discussed in Chapter 4. In this short review we will focus on that group of mental diseases in the field of Glu/GABA interactions; the insight into mechanisms of possible therapy will also be presented.

2. Glutamate-GABA turnover

The circle of GABA/Glu transformations is closed in the tripartite synapse, with no beginning and no end, as schematically shown in Fig. 2. In physiological conditions, the GABA vesicle content is in dynamic equilibrium with intraterminal glutamate concentrations (Mathews & Diamond, 2003).

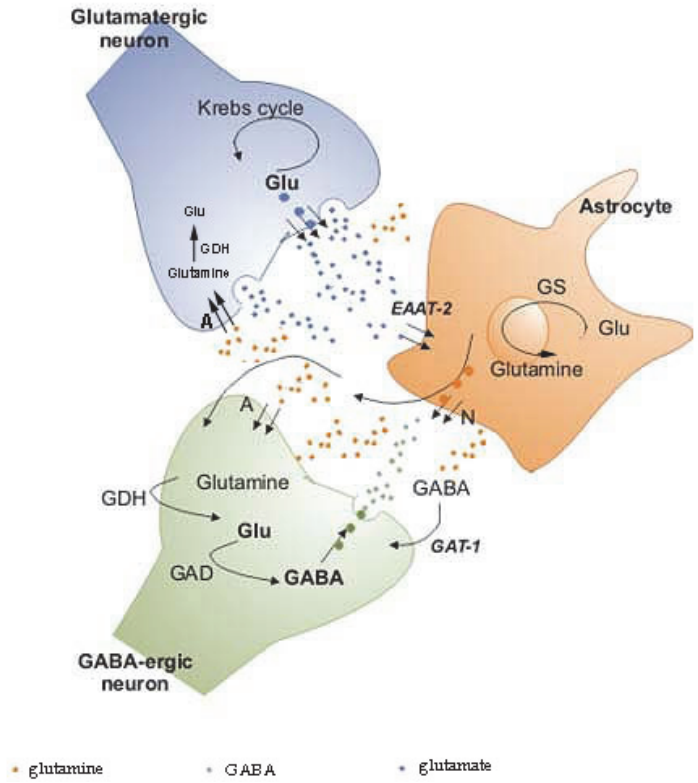

Fig. 2. The schematic presentation of glutamatergic-GABAergic transformation involving the pyramidal neuron, GABAergic interneuron and astrocyte. Glu-glutamate; GABA-γ amino butyric acid; GS-glutamine synthetase; GAD-glutamate decarboxylase; GDH-glutamine dehydrogenase; GAT-1-GABAergic transporter 1; EAAT-2-excitatory amino acid transporter 2; A-system A transporters; N-system N transporters

Glutamate is synthesized in neurons in the tricarboxylic acid circle (Liang et al., 2006) as well as in the glutamate-glutamine (GLX) cycle, which constitutes as an exogenous source of the neurotransmitter. In this cascade of events, glutamate, released from the presynaptic neuronal element, is transported to astrocyte through the EAAT-2 transporter. In the astrocyte, glutamine synthetase converts glutamate into glutamine, which is then transported into extracellular space through system N transporters, and is retrieved by the neuronal system A of amino acid transporters (Chaundhry et al., 2002). In neurons (both GABA and glutamatergic), glutamine is converted to glutamate in a reaction that is catalyzed by phosphate-activated glutamine dehydrogenase. In GABAergic inhibitory neurons glutamate further is converted into GABA by decarboxylation catalyzed with glutamic acid decarboxylase (GAD) (Liang et al., 2006). The inhibitory amino acid is then metabolized by transaminase to succinic semialdehyd and succinic acid, which re-enters the Kreb's cycle and is transformed into glutamate; the glutamate is released and uptaken by the astrocyte, and that closes up the cycle.
In the properly functioning CNS the release of neurotransmitters, and the neurotransmitters' effects evoked on target neuron is mediated by specific receptors.

3. Glutamate and GABA receptors

The neurotransmitter receptors of amino acids are split into several types, most broadly demarcated as ionotropic and metabotropic. Ionotropic receptors constitute as transmembrane ion channels that open or close in response to the binding of a ligand. These receptors convert the chemical signal of a presynaptically released neurotransmitter directly and very quickly into a postsynaptic electrical signal (Olsen & Sieghart, 2008), inducing the inhibitory postsynaptic potentials (IPSPs) or excitatory postsynaptic potentials (EPSPs), thus inhibiting or activating the neuron. Until now, two ionotropic receptors for GABA ($GABA_A$ and $GABA_C$) and three types of ionotropic receptors for glutamate (AMPA, KA, NMDA) have been discovered (Niswender & Conn, 2010; Olsen & Sieghart, 2008). The pharmacology of anxiety has been focused on $GABA_A$ receptors as the main site of action of ligands with anxiolytic activity. Type A of the GABA receptor is composed of five subunits of ligand-gated protein forming a pore selective to Cl^- anions. The subunits of the $GABA_A$ receptor constitute a relatively large family of several classes, including their splice variants (α1-α6; β1-β4; γ1- γ3, δ, ε, Θ, ρ1- ρ3). It enables the formation of a variety of combinations of specific subunits within the receptor, thus making it sensitive, or insensitive, to pharmacological manipulations (Millan, 2003; Olsen & Sieghart, 2008). Generally the receptor is a pentamer consisting of α, β and γ subunits in different combinations, and contains sites for the action of various endogenous and exogenous substances, such as neurosteroids, bicuculine, muscimol, benzodiazepines, ethanol, barbiturates and GABA (Olsen & Sieghart, 2008).
The NMDA receptor, one of the ionotropic receptors for glutamate, is both a ligand-gated and voltage-dependent heterotetrameric ion channel, consisting of two NR1 and two NR2 subunits (Conti et al., 1999). Activation of the receptor results in the opening of the non-selective channel to the cations. The receptor, similarly to $GABA_A$, possesses a variety of binding sites, such as the polyamine modulatory binding site, the Zn^{2+} modulatory binding site, glycine, glutamate, NMDA, MK-801 and phencyclidine binding sites (Danysz & Parsons, 1998). The schematic representation of $GABA_A$ and NMDA receptors with the most important binding sites present on each of them are shown on Figs. 3 and 4.

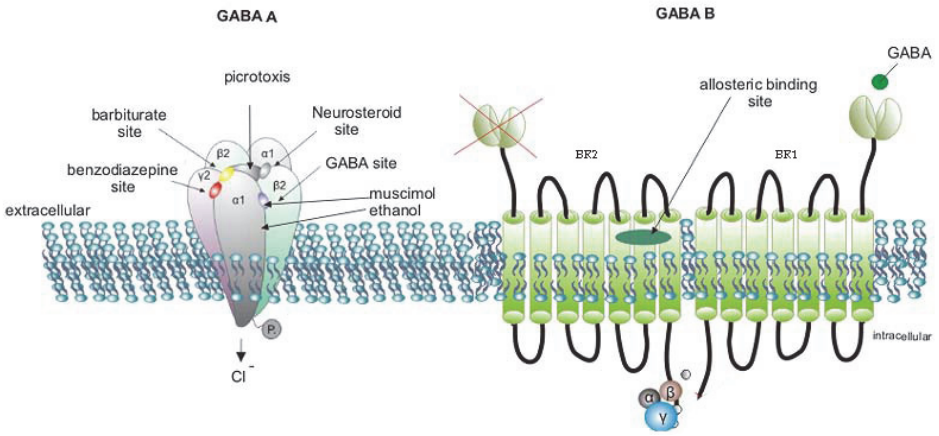

Fig. 3. The schematic representation of ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors for GABA. The five subunits of $GABA_A$ and their binding sites are shown on the left and the $GABA_B$ heterodimer composed of BR1 (binding a ligand) and BR2 (coupled to G proteins and possessing the allosteric binding site, but not binding the ligand) is shown on the right.

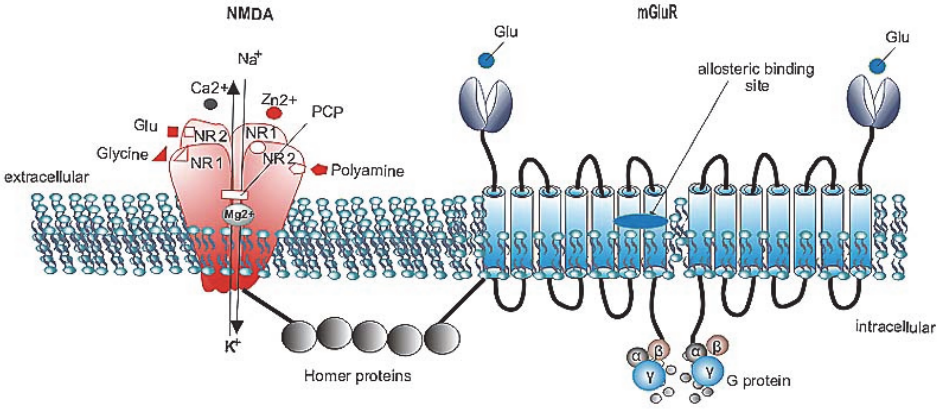

Fig. 4. The schematic representation of ionotropic (NMDA) and metabotropic (mGluR) receptors for glutamate. The four subunits of NMDA (NR1-NR2) and their binding sites are shown on the left and the mGlu homodimer composed of two identical parts of the mGlu receptor is shown on the right. Two orthosteric binding sites must bind a ligand to activate the receptor. An allosteric binding site is present within the 7 transmembrane domain.

By contrast, metabotropic receptors are connected with second messenger systems and exert a rather modulatory role in the CNS (Parmentier et al., 2002). Glutamatergic and GABA metabotropic receptors are linked to the G-proteins system and, opposite to the ionotropic ligand-gated channels, their action is slow and long-lasting (Bockaert et al., 2010). These receptors do not form an ion channel pore, although indirectly they can be linked to ion

channels through signal transduction mechanisms that induces the opening or closing of the channels (Ango et al., 2000). Metabotropic receptors on the presynaptic membrane can inhibit or, more rarely, facilitate neurotransmitter release from the presynaptic neuron (Schmitz et al., 2001). There is one metabotropic receptor for GABA ($GABA_B$ receptor) (Froestl, 2011) and 8 metabotropic receptors for glutamate (mGlu) (Pin and Duvoisin, 1995; Niswender & Conn, 2010). mGlu receptors are divided into three classes according to the sequence homology, pharmacology and the second messenger system they activate. The schematic classification of mGlu receptors is shown in Table 1.

The typical G protein-coupled receptor consists of seven hydrophobic transmembrane domains linked with extra- and intracellular loops, with the N terminus located on the extracellular side of the membrane and C terminus on the intracellular side (Parmentier et al., 2002). Both $GABA_B$ and mGlu receptors belong to the III family of the G protein-coupled receptors (GPCRs). The characteristic feature of this class of receptors is the forming of obligatory, functional dimers, possessing a large extracellular ligand-binding domain which closes up like a venus-flytrap after binding a ligand (Figs. 3 and 4) (Pin Duvoisin, 1995; Niswender & Conn, 2010; Bockaert et al., 2010).

mGluR	Group I positively coupled to phosphatydylo inosytol	mGluR1	a, b, c, e
		mGluR5	a, b
	Group II negatively coupled to adenylyl cyclase activity	mGluR2	
		mGluR3	
	Group III negatively coupled to adenylyl cyclase activity	mGluR4	a, b
		mGluR6	a, b, c
		mGluR7	a, b, c, d, e
		mGluR8	a, b, c

Table 1. The classification of mGlu receptors (Wierońska &Pilc, 2009)

The ligands of the GABAergic or glutamatergic receptors were shown to possess excellent anxiolytic activity (for review see: Pałucha & Pilc, 2007; Froestl, 2011). Below, the hypothesis of the possible mechanisms by which ligands of the receptors for two amino acids restore the lost in the anxiety inhibitory/excitatory balance in the CNS will be presented . But, firstly, a few words on anxiety.

4. Anxiety

The pathophysiology of anxiety disorders is a complex phenomenon and to designate one direct cause of their origin is almost impossible. However, those disorders recently became a serious public problem as a growing percentage of the population is being diagnosed with anxiety every year, and it is the most prevalent mental health problem in Europe and the United States (Wittchen & Jacobi, 2005). According to the classification of psychiatric disorders, the term Anxiety Disorders covers nearly 12 different pathological states (DSM-V), including panic disorder, generalized anxiety disorders, post-traumatic stress disorder, social phobia, specific phobias. These mentally ill people are then mainly excluded from

normal life for months or even years. Therefore, the search for effective and safe medicine is one of the goals of present neuropharmacology. The effectiveness of the drugs depends, largely, on the mechanism of their action. Thus, the important thing is to find out the functionally disrupted pathways in the CNS leading to neuropsychiatric illnesses and to indicate the possible targets for searching new psychotropic drugs. When it comes to anxiety disorders, the involvement of different neurochemical pathways were discussed during the past few decades.

Several neurotransmitters mediate the different components of anxiety, including excitatory amino acids such as Glu and inhibitory such as GABA. Generally, different aspects of anxiety response are mediated by various neurotransmitters in anatomically distinct areas. Important for our consideration dynamic balance between inhibitory/excitatory forces in the brain is thought to be disrupted with increased excitation leading to anxiousness. An increase in the glutamate efflux in the prefrontal cortex and hippocampus was observed after stress (Moghaddam et al., 1993; Bagley & Moghaddam, 1997). Anxiogenic behavior was observed in mice lacking the GAD65, enzyme responsible for converting Glu into GABA (Kash et al., 1999).

5. Present anxiolytics and future perspectives

In the pharmacological treatment of anxiety, drugs with a different mechanism of action are available. These include benzodiazepines, 5-HT1A agonists, and antidepressant medications. They all have their advantages and disadvantages, but as the review concerns GABAergic and glutamatergic neurotransmission, the compounds involving other mechanisms of action will not be discussed here, as they are widely described elsewhere (see: Millan, 2003).

The most efficacious anxiolytic drugs are the positive modulators (PAM) acting at the benzodiazepine binding site on the $GABA_A$ receptor, thus enhancing the affinity of the natural agonist to the receptor, known as benzodiazepines (Sternbach et al., 1974). The number of representatives of the group reaches nearly 80, and diazepam is probably the best known not only as an anxiolytic, but also as a hypnotic drug. Although the drugs have relatively good efficacy, a variety of adverse effects is also described. The most common are: ability to induce tolerance, sedation, myorelaxation, and dependence (Millan, 2003). Moreover, memory impartment and interaction with alcohol can occur. That is supposed to be connected with the activation of the α1 subunit of the $GABA_A$ receptor (Esclapez et al., 1996). The other binding sites of the receptor, such as barbiturates, muscimol or picrotoxin (shown on Fig. 3) are even worse drug targets. Although the anxiolytic-like activity of benzodiazepines is connected with activation of α2-α3 subunits, the majority of drugs activate to some extent the other subunits, too (Gao et al., 1993; Esclapez et al., 1996), thus being responsible for variety of adverse effects that may occur.

The discovery of the metabotropic $GABA_B$ receptor brought new possibilities for searching agents with the mechanism of action based on the enhancement of GABA transmission. Because of the relatively short time since the cloning of the receptor (which was in the year 1997), the clinically effective drug activating the receptor with anxiolytic efficacy are lacking at present; the orthosteric agonist of the receptor, baclofen, introduced in 1977 for the treatment of multiple sclerosis (Sachais, 1977), induces a variety of adverse effects including sedation and miorelaxation, whilst the anxiolysis was not discussed as an asset of the drug. However, in 2000 the first positive modulators of the $GABA_B$ receptor were discovered. The

preclinical trials were promising as all of the compounds possessed anxiolytic activity and were free of adverse side-effects typical for benzodiazepines, such as sedation or miorelaxation (Froestl, 2011). Interestingly, the antagonist of $GABA_B$ receptors are not active as anxiolytics, being rather described as possible antidepressants (Pilc & Nowak, 2005). Table 2 summarizes the main classes of the compounds activating GABA receptors, with special attention to their anxiolytic efficacy.

$GABA_A$ ligands	**benzodiazepine site PAMs** (benzodiazepines)	see: Millan, 2003
	GABA transaminase inhibitors (γ-vinyl GABA, aminooxyacetic acid)	Sherif et al., 1994
	GABA reuptake inhibitors (tiagabine)	Schaller et al., 2004
	GABA agonists (muscimol, THIP)	
	ethanol	Corbett et al., 1991
	neurosteroides	see: Millan 2003
$GABA_B$ ligands	**positive allosteric modulators** (GS39783, CGP7930, CGP13501, NVP-BHF177, (+)-BHFF)	see: Froestl, 2011

Table 2. GABAergic ligands with anxiolytic activity.

Pharmacological investigation of the glutamatergic system had lagged far behind research into the GABA systems because of the limitations connected with the use of ionotropic receptors ligands. Although some of the compounds acting at the NMDA and AMPA receptors were shown to possess anxiolytic activity, the adverse effects after the administration of antagonists of those receptors, such as the psychotomimetic effects and influence on locomotor activity were observed (Danysz & Parsons., 1998). The narrow window between therapeutic doses and doses inducing adverse effects caused a quick end to the therapeutic hopes connected with that receptor. However, it did not shatter the glutamatergic system as a target for anxiolytic drugs. A few clinical trials showed that some commonly used medications were found to exert their therapeutic effect by modulating glutamatergic transmission (*via* the inhibition of voltage-dependent ion channels). Additionally, these compounds were shown to be effective in anxiolytic disorders in randomized, double-blind, placebo-controlled trials (Table 3).

Compound	Anxiolytic activity tested in the clinic
Pregabalin	generalized anxiety disorder
Topiramate	post-traumatic stress disorder, specific phobias
Lamotrigine	post-traumatic stress disorder
Riluzole	generalized anxiety disorder
Tiagabine	generalized anxiety and post-traumatic stress disorder
Valproic acid	panic disorder, social phobia
Phenytoine	post-traumatic stress disorder
Gabapentin	social phobia
Levetiracetam	specific phobias, panic disorder
D-cycloserine	post-traumatic stress disorder, phobia

Table 3. Examples of anxiolytic-like activity of agents modulating glutamatergic activity (Amiel &Mathew, 2007).

The discovery of metabotropic glutamate receptors opened a broad range of possibilities to modulate the glutamatergic system. They became a target for putative anxiolytics, including antagonist, agonist or modulators (depending on the type of receptor they bind). A variety of subtypes involving different second messenger systems, with an expression in all of the brain regions both post- and presynaptically as auto– and heteroreceptors, make those receptors a very attractive therapeutic target. A number of preclinical studies clearly indicated that ligands of those receptors are excellent anxiolytics (Pałucha & Pilc, 2007; Wieronska & Pilc, 2009). Especially interesting agents were found among antagonists of the first group and agonists of the second group of mGlu receptors (Wieronska & Pilc, 2009). The clinical studies, which started in early '90s of the last century with fenobam, a drug with a mechanism of action that was unknown at the time, revealed that the compound was evidently effective as a novel, non-benzodiazepine anxiolytic (Porter et al., 2005). Today, we know that the drug is a negative allosteric modulator of the mGlu5 receptor. Similarly, the mGlu2/3 agonists have undergone positive clinical trials, such as LY354740 and its derivative, LY544344 (Dunayevich et al., 2008).

NMDA ligands	**NMDA channel blockers** *(memantine, MK-801)* **competitive antagonists** *(L-AP4, L-AP7, MDL100453, CGP37849, CGP39551, NPC17742)* **inverse agonists** *(ACPC)* **glycine site antagonists** *(5,7 dichlorokinurenic acid, L 701324)*	see: Danysz &Parsons, 1998
AMPA ligands	**antagonists** *(LY326325, LY382884, LY293558)* **2,3 BZD AMPA site antagonists** *(GYKI52466, GYKI53404, GYKI53655, EGIS8332, EGIS9637, EGIS10608)*	Alt et al., 2007 Kapus et al., 2008
mGlu ligands	**mGluR1 antagonist** *(JNJ16259685, AIDA, LY456236, EMQMCM)* **mGlu5 NAMs** *(MPEP, MTEP)* **mGlu5 antagonist** (fenobam) **mGlu2/3 agonists** *(LY 354740, LY 314582, LY 544344, LY 404039, LY 379268)* **mGlu2 PAMs** *(4-APPES, CBiPES, BINA, LY487379)* **mGlu2/3 antagonists** *(MGS0039, LY341495)* **mGlu4 agonist** *(LSP1-2111, ACPT-I)* **mGlu7 PAM** *(AMN082)*	see: Wierońska &Pilc, 2009 see: Pałucha &Pilc, 2007 Porter et al., 2005 Linden et al., 2005, 2006; Dunayevich et al., 2008 see: Wierońska &Pilc, 2009 Iijima et al., 2007 Stachowicz et al., 2008, 2009, Wierońska et al., 2010

Table 4. Glutamatergic receptors ligands with anxiolytic activity.

The third group of mGlu receptors is the biggest one and has been the least investigated so far, mainly because of the lack of selective and brain-penetrating agents. ACPT-I was the first brain penetrating compound, activating both mGlu4 and mGlu8 receptors. The compound exerted an anxiolytic-like efficacy in rodents (Stachowicz et al., 2009). Later on, a more selective compound, LSP1-2111 was synthesized, and was shown to preferentially activate mGlu4 receptors. Anxiolytic-like activity was described after the administration of relatively low doses (Wieronska et al., 2010). The glutamatergic receptors ligands with anxiolytic activity are listed in Table 4.

Taken all together it appears that, as stated above, both glutamatergic and GABAergic agents may evoke anxiolysis, mainly through agonistic action. Below the mechanism of action of those ligands will be introduced with an indication of a common direction of action leading to inhibition of the excessively active glutamatergic system.

6. Mechanism of action of GABAergic agents

The involvement of the GABAergic system, in particular the action of GABAmimetics stimulating GABA receptors, such as benzodiazepines, is a certainty in the present neuropharmacology of anxiety. The general mechanism of action of those compounds is an enhancement of GABAergic neurotransmission in the brain, which is tantamount to the enhancement of inhibition; however, the point is not in the inhibition per se but, rather, in the cascade of events caused by the inhibition.

As was mentioned above, GABA acts through three different types of receptors. As the pharmacology of the ionotropic $GABA_C$ receptor is the least investigated at present, we will focus on the mechanism of action of the ligands of two others: the ionotropic $GABA_A$ and metabotropic $GABA_B$ receptor (Froestl, 2011). Activation of both receptors causes an inhibition of neuronal excitability. However, what it means exactly depends on the expression of these receptors on the type of neuron.

6.1 $GABA_A$ signaling

Typical anxiolytic drugs, benzodiazepines, act by enhancing the inhibitory effects of GABA at $GABA_A$ receptors containing either an α_1, -2, -3 or -5 subunit. Postsynaptic expression of $GABA_A$ receptors composed of responsible for the anxiolytic-like efficacy α2 - α3subunits was shown mainly on GAD-positive neurons, that is GABAergic interneurons (Gao et al., 1993; Esclapez et al., 1996). Therefore, activation of those $GABA_A$ receptors [see Fig.5 (1)] would inhibit the GABAergic neurotransmission. Moreover, such an inhibition would, in turn, exert anxiolysis only indirectly, possibly through the disinhibition of the GABAergic projection neuronal element, increasing an inhibitory action on pyramidal target neurons. The described mechanism is supposed to be responsible for inhibiting glutamatergic neurons in structures mediating anxiolytic response, such as the lateral amygdala (Rainnie e al., 1991), and medial prefrontal cortex (mPFC) (Gigg et al., 1994).

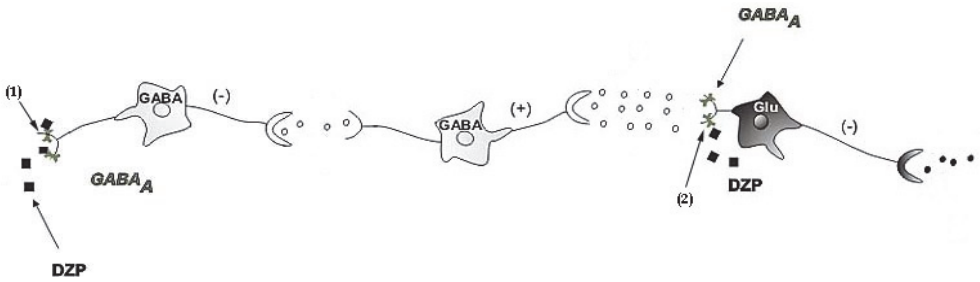

Fig. 5. The schematic neuronal network showing the mechanism of the postsynaptic action of the $GABA_A$ agonist, diazepam (see description in the text). empty dots- GABA; black dots-Glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released

The GABA released from inhibitory interneurons, and GABAmimetics administered exogenously, may also inhibit the glutamatergic pyramidal neurons *via* $GABA_A$ receptors expressed on the dendrites and soma of pyramidal neurons [see:Fig.5 (2)]. The activation of these receptors would directly lead to the inhibition of excitatory amino acid neurotransmission, as was shown in the electrophysiological studies on IPSPs in the glutamatergic neurons of the basolateral amygdala (BLA) (Chhatwal et al., 2005), as well as in the pyramidal cells of the piriform cortex and hippocampus (Samulack et al., 1993; Kapur et al., 1997).

Although the inhibitory effect of GABA mediated through the $GABA_A$ receptor is commonly considered to be postsynaptic, the presynaptically expressed $GABA_A$ receptors were also described in the variety of neurons in the CNS; however, the pharmacological properties of those receptors are relatively poorly understood. Mossy fibers in the hippocampus representing the axons of granule cells constitute one of the sites of the presynaptic expression of GABA receptors (Jang et al., 2006). Activation of these receptors induces neuron depolarization and facilitates spontaneous glutamate release (Jang et al., 2006). The standard anxiolytic drug, diazepam, was shown to induce an increase in the frequency of EPSPs and the potentiation of muscimol-induced glutamate release (Han et al., 2009). Although at first sight the effect may seem paradoxical, it may fit the theory of diazepam-mediated anxiolysis when considered through a variety of histological and electrophysiological data. The axons of granule cells synapse with a wide variety of inhibitory GABA interneurons in the hilar region of the dentate gyrus before continuing on to innervate pyramidal cells in the CA3 region. Therefore, the increased glutamate release by presynaptically active diazepam [see: Fig.6 (1)] would activate GABAergic interneurons which would then go on to inhibit increased excitation and thus lead to anxiolysis. Therefore, the circle closes up as the excitation leads to inhibition and inhibition inhibits the excitation.

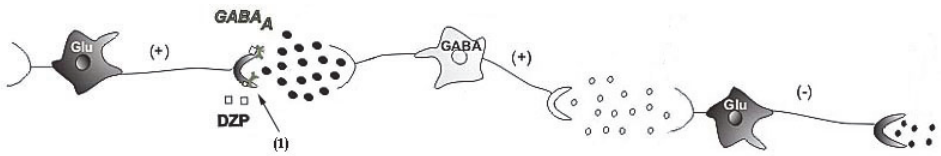

Fig. 6. The schematic neuronal network showing the mechanism of the presynaptic action of the $GABA_A$ agonist, diazepam (see description in the text). empty dots- GABA; black dots-glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released

6.2 $GABA_B$ signaling

As an alternative to the ionotropic $GABA_A$ receptor, there is the $GABA_B$ receptor that is capable of exerting the slow and modulatory action of inhibitory neurotransmission, often in close association with the $GABA_A$ pentamer (Kardos et al., 1994). Similar to the above described ionotropic channel, the $GABA_B$ receptor was shown to be expressed both pre- and postsynaptically. Presynaptically expressed heterodimers are generally composed of $GABA_{B1A}$/$GABA_{B2}$ subunits, while postsynaptic neurotransmission is mediated by the $GABA_{B1B}$/$GABA_{B2}$ tandem (Billinton et al., 1999).

The $GABA_B$ receptor has only been cloned relatively recently, so the well documented clinical trials concerning the anxiolytic activity of its ligands are poorly available. However, based on the electrophysiological, histochemical and behavioural studies presenting its ability to balance the excitatory/inhibitory forces in the CNS, it is very likely that it may become a promising target in the search for novel anxiolytics. In the hippocampal slices, the subpopulation of interneurons was selected that inhibits pyramidal cells *via* $GABA_B$ postsynaptic receptors (Samulack et al., 1993; Forti et al., 1997), independently on $GABA_A$ signalling. Therefore, pharmacological stimulation of the $GABA_B$ receptor on pyramidal neurons would exert the inhibitory effect on glutamatergic transmission, thus inducing anxiolytic efficacy [see: Fig. 7 (2)].

Besides the inhibitory influence on pyramidal cells, $GABA_B$–mediated inhibition was also observed on inhibitory interneurons, when measured with whole cell patch-clamp techniques (Mott et al., 1999). Pharmacological stimulation of these receptors would inhibit the inhibition [see: Fig.7 (1)]. As described for $GABA_A$ receptor activation, such action exerts anxiolysis only after the disinhibition of GABAergic network innervating target pyramidal neurons.

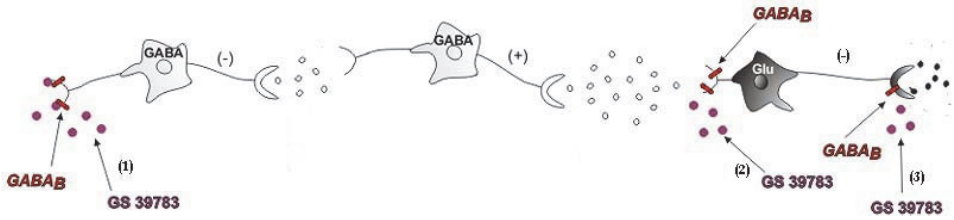

Fig. 7. The schematic neuronal network showing the mechanism of the postsynaptic action of the $GABA_B$ receptor positive allosteric modulator, GS39783 (see description in the text). empty dots- GABA; black dots-Glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released

The speculation on the mechanism of putative anxiolysis mediated by activation of the $GABA_B$ receptor does not end on the postsynaptic effects. Heterodimers were shown to be localized presynaptically along the extrasynaptic plasma membrane of axon terminals and along the presynaptic active zone in both asymmetrical and, to a lesser extent, symmetrical synapses (Lopez-Bendito, 2004). The fundamental role of these receptors is the inhibition of the release of the neurotransmitter [see: Fig.7 (3)]. The majority of presynaptically expressed $GABA_B$ heteroreceptors was found on the glutamatergic nerve terminals. The activation of these receptors would inhibit the excitatory amino acid release, resulting in anxiolysis.

7. Mechanism of action of glutamatergic agents

The vast diversity of receptors for glutamate creates a variety of possibilities to influence both excitation and inhibition in the brain. The age of glutamate began with the discovery of metabotropic glutamate receptors in 1986, which shortly became a promising alternative to iGlu receptors in a variety of investigations in the field of neuropharmacology (Nicoletti et al., 1986). Presently, the important role of mGlu receptors in anxiety is almost unquestioned and their role as the important anxiolytic drug targets is well established. However, the role of ionotropic receptors, NMDA and to some extent AMPA, is still significant despite the limitations connected with adverse effects induced by their ligands. The expression of the receptor was detected on dendritic terminals of glutamatergic neurons and interneuronal

post-synaptic sites, thus influencing the firing of both inhibitory and excitatory projections (Conti et al., 1999; Standaert et al., 1999; Ratzliff et al., 2001). The variety of different combination of NR1-NR2 subunits results in the existence of the diversity of receptor variants, expressed differently on a subpopulation of neurons and affecting function and selective vulnerability (Landwehrmeyer et al., 1995; Standaert et al., 1999). It creates a potential for altering the balance of inhibition and excitation independently in selected parts of the brain. For example, in the amygdale, the structure known as the responsible for storage of fear memories, NMDA, composed of NR1-NR2B subunits, was shown to be expressed mainly in the synapses of the central nucleus, while in the lateral nucleus the receptor contains both NR2A and NR2B subunits (Sah et al., 2003). It remains open for further investigation whether or not it has some functional meaning.

As was mentioned above, the iGlu receptors will not be discussed here as a putative target for new drugs. However, it is worth mentioning that the blockers of the NMDA receptor were shown to possess anxiolytic-like activity. One of the compounds, memantine, was effective in humans and has undergone successful clinical trials (Aboujaoude et al., 2009). In the preclinical studies, the antagonists of the second iGlu receptor, AMPA, exerted anxiolytic-like efficacy as well, supporting the important role of the iGlu receptors in anxiety (Kapus et al., 2008). The AMPA expression was predominantly found on pyramidal cells and interneurons, among others in the amygdale, known for its role in stress response (Sah et al., 2003). To all the possibilities the action of memantine is mediated *via* the NMDA receptor localized postsynaptically on inhibitory interneurons. Blockade of those receptors by the antagonist [see: Fig.8 (1)] would inhibit the GABAergic tone which would contribute to the stimulatory effect on inhibition followed by the inhibition of excitation.

This experimental data clearly shows that the blockade of the receptors exerts anxiolytic function. In the physiological conditions, the endogenous antagonists are not available or, at least, are not identificable, so the activity of the receptors is regulated predominantly by the glutamate. The level of amino acid regulates both the anxiety state and the anxiolytic response. As the iGlu receptor was shown to be expressed by the postsynaptic membrane of pyramidal neurons, the increased level of glutamate would lead directly to depolarization of the neuron and the activation of glutamatergic network activity, inducing an elevated stress response. Therefore, the anxiolysis could be induced by decreasing the level of endogenously released glutamate. Diminished glutamate release would activate the NMDA receptor to a lesser degree and the excitation of the CNS would remain at a stable level. Such an effect can be achieved by switching on the regulatory machinery of presynaptic glutamate release. mGlu receptors contribute to the effect, being the most important pawns in the circle.

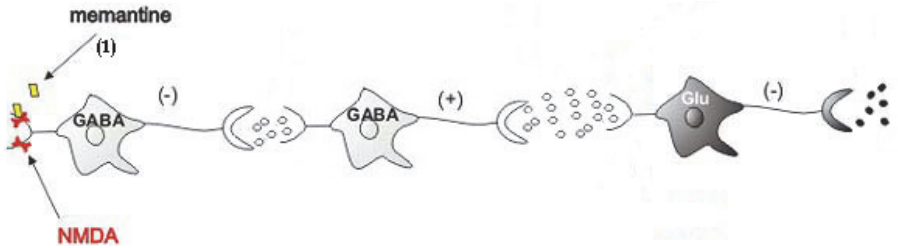

Fig. 8. The schematic neuronal network showing the mechanism of action of the NMDA receptor antagonist, memantine (see description in the text). empty dots- GABA; black dots- Glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released

Below, step by step, the possible mechanisms of the anxiolytic-like efficacy of metabotropic glutamate receptor ligands will be described, paying special attention to the excellent regulatory action exerted by the II and III group of the receptors both in asymmetrical and symmetrical synapses. The dynamically changing synaptic cleft environment and the amount of available neurotransmitters are dependent on their proper functioning. By contrast to the II and III groups of the receptors, the representatives of the first group tend to mediate the postsynaptic action which itself mediates the slow excitatory current (Pin & Duvoisin, 1995). From this group our deliberations start.

7.1 Group I mGlu receptors

Although the first group of receptors is predominantly distributed on the post-synaptic parts of the neurons, the presynaptic localization was also described.

The ligands of these receptors, especially the antagonists of the mGlu5 subtype, were shown to possess profound anxiolytic activity. A variety of preclinical experiments with negative allosteric modulators of the receptor, MPEP and MTEP, were further confirmed in the clinical studies, when fenobam, a mGlu5 antagonist, was first described as the non-benzodiazepine anxiolytic drug (see: Pałucha&Pilc, 2007; Porter et al., 2005).

The antagonistic action of MPEP, and probably other ligands acting at the mGlu5 receptor, results in the inhibition of stimulated DHPD PI hydrolysis and the neuronal firing in the CA1 area of the hippocampus (Kuhn et al., 2002), generally inducing the inhibition of the target cell. Immunohistochemical studies at the electron microscopy level indicate that mGlu5 receptors form functional oligoheteromers with NMDA receptors, and the group of Homer proteins is responsible for coupling mGlu5 with NMDA (Ango et al., 2000). Electrophysiological and biochemical studies confirm the functional dependence between these two receptors, as the NMDA-mediated current and NMDA-induced increase in the CREB phosphorylation were reduced by MPEP (Lindemeyer et al., 2006). The above findings characterize the inhibitory nature of the mGlu5 antagonists. However, the mechanism of the anxiolytic-like efficacy of the compounds involves the target neuronal elements expressing the receptor. Based on electrophysiological and immunohistochemical data, mGlu5-NMDA complexes are expressed predominantly on the inhibitory interneurons in the hippocampus (Sanon et al., 2010), cortex (Sarihi et al., 2008) or amygdala. As such, it would appear that the MPEP-induced inhibitory action on GABAergic interneurons is responsible for its anxiolytic effect [see: Fig.9 (1)]. This inhibition of the inhibition results in

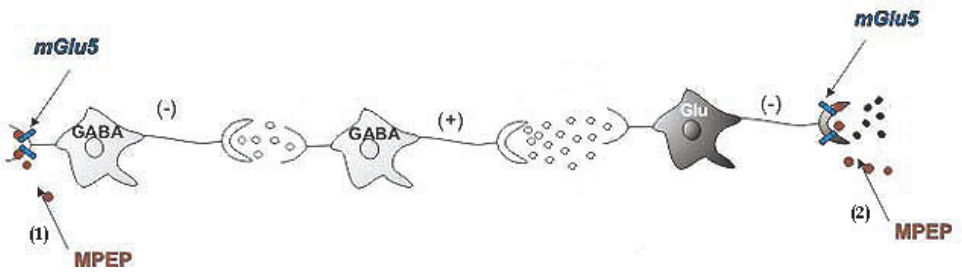

Fig. 9. The schematic neuronal network showing the mechanism of action of the MPEP, mGlu5 receptor antagonist (see description in the text). empty dots- GABA; black dots-Glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released

a rich repertoire of changes which take place the synapses between interneurons and pyramidal neurons to induce feedback inhibition of increased excitation. Indeed, as microdialysis studies indicate that the level of glutamate, yet not GABA, is decreased after MPEP administration, at least in periagueductal gray, one of the structures involved in anxiety response (de Novellis et al., 2003).
Although it seems that anxiolysis induced by mGlu5 antagonists is mediated mainly through their action at the postsynaptic site, the presynaptic expression of group I mGlu receptors was also described. mGlu5 labelling at axon terminals was not so intensive as on neuronal somata, but was observed in some glutamatergic axonal terminals (Rae et al., 2004). The blockade of these receptors would inhibit the release of glutamate, causing anxiolytic response [see: Fig.9 (2)].

7.2 Group II mGlu receptors

The second group of metabotropic glutamate receptors involves mGlu2 and mGlu3 subtypes, negatively stimulating adenylyl cyclase activity (Niswender & Conn, 2010). The majority of the agonist and positive modulators of these receptors possess excellent anxiolytic efficacy. Among the available ligands it is hard to find one not showing such activity in the preclinical studies. As the agonists mostly activate both subtypes of the receptors, the estimation of the independent participation of each subtype in anxiolysis is difficult. Selective positive modulators of the mGlu2 subtype exert anxiolytic-like efficacy indicating the important role of the receptor in mGlu2/3 agonist-mediated anxiolysis. However, the role of the second subtype is more enigmatic because of the lack of selective, brain penetrating agents acting on the mGlu3 subtype. Some indirect conclusion can be drawn on the basis of the results obtained with the use of mGlu2 knockout mice. The majority of effects observed after administration of mGlu2/3 agonists were lacking in these animals, suggesting the mGlu2-dependent action of ligands (Linden et al., 2006; Woolley et al., 2008). Although some controversial results showing the involvement of the mGlu3 receptor in mGlu2/3-mediated anxiolysis can also be found (Linden et al., 2005), the activation of the mGlu2 receptor seems to be crucial for mGlu2/3 agonists-mediated anxiolysis.
Among all of the mGlu receptor subtypes located in structures connected with fear response, mGlu2 receptor seems to be expressed predominantly on glutamatergic terminals, in pre-terminal rather than terminal portions of the axons (Petralia et al., 1996; Shigemoto et al., 1997). The expression of the mGlu2 receptors, as shown in the diagram, suggests that activation of the receptor occurs during abnormal and elevated glutamate release, allowing the neurotransmitter to regulate its own release. As the receptors are not in close association with glutamatergic synapses and a subpopulation of the receptors not associated with any synaptic junction was identified, the receptor can be probably activated by glutamate of a nonsynaptic origin. The astrocytes constitute the main source of this additional glutamate pool in the CNS [as shown on Fig.10]. The glutamate released by single astrocyte onto adjacent neuronal processes controls simultaneously the excitability of several neighboring pyramidal cells (Angulo et al., 2004), and the mGlu2 receptor could play an important role in this process. Besides this, a growing line of evidence indicates that glutamate is able to escape the synapse from which it is released and diffuse into neighboring junctions to activate receptors there (Diamond, 2002). The occurrence of this type of heterosynaptic inhibition was demonstrated at mossy fibre synapses in the hippocampus (Vogt & Nicoll, 1999), the place where there is rich mGlu2 innervation (Petralia et al., 1996). Exogenously

administrated compounds acting on these receptors would restore the twisted excitatory/inhibitory balance independently on synaptic machinery, playing the supportive role in the self-regulating circle, which can itself be disrupted in a pathological state.

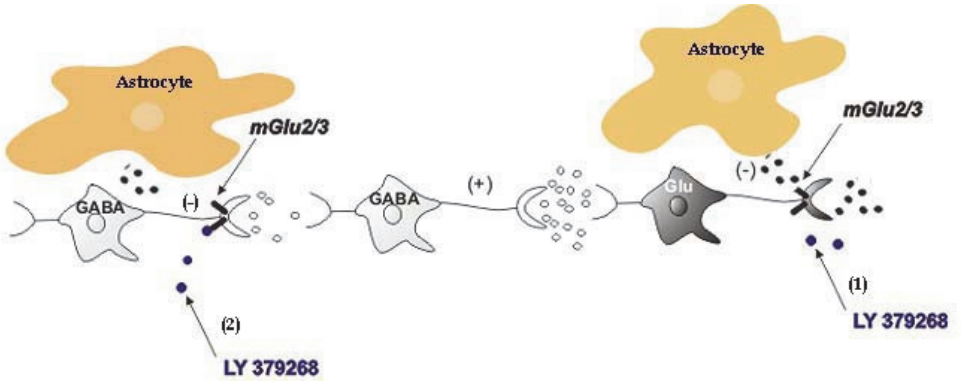

Fig. 10. The schematic neuronal network showing the mechanism of the action of mGlu2/3 receptor agonist, LY379268 (see description in the text). empty dots- GABA; black dots-Glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released.

By contrast to group I and group III mGlu receptors (which will be discussed later), mGlu2 agonists directly inhibit glutamatergic neurotransmission, in majority not involving any other neurotransmission systems *via* presynaptic blockade of glutamate release [see: Fig.10 (1)]. In electrophysiological studies, a direct inhibition of projecting basolateral neurons, and the reduction of the excitatory drive were observed after administration of LY354740 and LY379268, mGlu2/3 receptor agonists (Muly et al., 2007). The inhibitory effect on stimulated glutamate release was also observed in microdialysis studies (Xi et al., 2002; Johnson et al., 2005).

However, some studies indicate that the release of GABA in the hippocampus stays under inhibitory control of group II mGlu receptors expressed on GABAergic terminals [see: Fig.10 (2)] (Kogo et al., 1999). If this regulatory action on inhibition contributes to anxiolysis, the intermediary interneuron innervating pyramidal glutamatergic target cells must be disinhibited.

7.3 Group III mGluR

The third group of metabotropic glutamate receptors constitutes the largest family and involves mGlu4, mGlu6, mGlu7 and mGlu8 subtypes. The most important for our consideration are mGlu4 and mGlu7 representatives, as the ligands of these receptors exerted clear anxiolytic action. mGlu6 expression is restricted mainly to the retina (Laurie et al., 1997) and the mGlu8 selective and brain-penetrating ligands have been poorly available so far. The studies concerning the recently synthesized positive allosteric modulator of the receptor, AZ12216052, indicate that stimulation of the receptor could result in anxiolysis (Duvoisin et al., 2010).

Expression of the group III mGlu receptors subtypes is distinct and somewhat complementary throughout the structures involved in anxiolysis. The receptors are, above

all, presynaptic and are usually located close to the center of the synaptic cleft. The receptors show a highly selective expression and subcellular location on nerve terminals modulating neurotransmitter release. Contrary to the other described presynaptic auto- or heteroreceptors, the stimulation of the receptors of the third group decreases not only vesicular and non-vesicular glutamate release (Xi et al., 2003), but also depresses the release of GABA from interneurons (Rusakov et al., 2004).

The mGlu4 receptor can act as autoreceptor expressed by glutamatergic terminals or as the heteroreceptor localized on GABAergic axons, suggesting the role of the mGlu4 receptor in in the regulation of both types of neurotransmitter (Corti et al., 2002). The excitatory input to the hilar-dentate border of the interneurons was depressed after mGlu4 receptor activation (Doherty & Dingledine, 1998). This may result in the disinhibition of GABA-releasing terminals that innervate the principal cells [see: Fig.11 (1)], thus inhibiting glutamatergic network.

The presence of mGlu4 receptors on hippocampal interneuronal terminals projecting from the hilus was also described, and with the use of electrophysiology it was shown, that they innervate the other GABAergic postsynaptic element [see: Fig.11 (2)] (Kogo et al., 2004). The source of excitatory amino acid in this kind of GABA-GABA synapse may come from glutamate spillover, allowing for the heterosynaptic regulation of the functional excitatory/inhibitory network. The disinhibition of the postsynaptic interneuron would regulate the activity of target glutamatergic cells.

Among all of the mGlu group III receptors, mGlu4 receptor revealed a relatively high level of post-synaptic staining, confirmed both in light and electron microscopy studies on pyramidal neurons in the some areas of the hippocampus (Bradley et al., 1996). Whether the anxiolytic-like action of mGlu4 receptor agonists involves the activation of these post-synaptically expressed receptors, however, still remains open for discussion.

The other candidate for regulating the glutamate/GABA level in the CNS is the mGlu7 receptor, widely distributed through the CNS, in the pre-synaptic grid, at the site of the synaptic vesicle fusion (Shigemoto et al., 1996). The axon terminals expressing the mGlu7 receptor were observed to be concentrated densely and specifically on mGluR1α-like immunoreactive GABAergic interneurons [see: Fig.11 (3)] (Shigemoto et al., 1996; Kinoshita et al., 1998). Therefore, the final result of the pre-synaptic action of the activated mGlu7 receptor is modulation of the postsynaptic GABAergic target [see: Fig.11 (3)]. This inhibition would cause the disinhibition of the other interneurons, targeting the glutamatergic network. The pyramidal neurons expressing mGlu7 on their terminals can form synapses with dendrites of the pyramidal cells; however, the expression of the mGlu7 receptor was found to be almost ten-fold higher in these pyramidal axons that innervate the mGluR1 alfa-expressing interneurons (Samogyi et al., 2003). Interestingly, mGlu7 receptors are also expressed on some types of the interneuron population (e.g VIP positive) innervating mGlu1α-somatostatine postsynaptic interneurons [see: Fig.11 (4)] (Dalezios et al., 2002) and creating a kind of GABA-GABA synaptic junction. Similar to the one described for mGlu4, the mechanism of anxiolysis involves inhibition of the GABA release, and in the simplest scenario, the depression of the GABA release could lead to a disinhibition of postsynaptic interneuron and increased GABA release on their terminals, inhibiting the input zone to the pyramidal cells. However, as the affinity of the mGlu7 receptor to glutamate is very low, in these kind of symmetrical GABAergic synapses to all the possibility the receptor is not activated by endogenous glutamate. mGlu7 PAM can possibly sensitise the affinity of the

receptor to glutamate, leading to anxiolysis. In rare cases, glutamate and GABA can be stored and released by the same nerve terminals (Walker et al., 2001), although in a properly functioning brain the glutamate is metabolized to GABA in interneurons. As the impartment in GAD67, the enzyme responsible for catalyzing the reaction was described in mood disorders and it can be speculated that under pathological conditions the glutamate is released by GABAergic interneurons, thus activating presynaptic glutamatergic receptors (Kash et al., 1999).

The distribution of the mGlu8 receptor was observed on the presynaptic active zones of neurotransmitter release on identified GABAergic and putative glutamatergic terminals that create synapses with several types of GABAergic neurons (Ferraguti et al., 2005). The type of the synapse predicts a role in adjusting the activity of interneurons depending on the level of network activity, widely described several times before now. The receptor is often expressed closely to its mGlu7 relative, therefore its action would involve similar mechanisms to those described above.

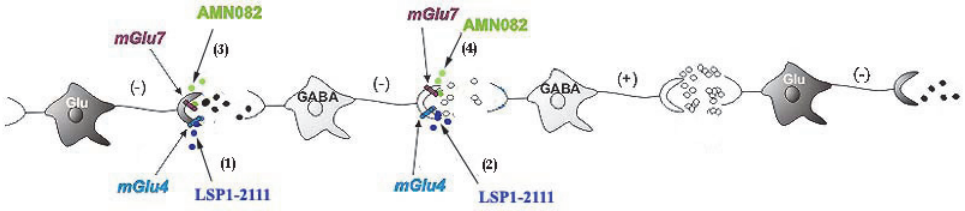

Fig. 11. The schematic neuronal network showing the mechanism of action of the mGlu group III receptor agonist LSP1-2111 (mGlu4 agonist) and AMN082 (mGlu7 agonist) (see description in the text). empty dots- GABA; black dots-Glu; (-)-inhibition; (+)-enhancement; the number of dots indicates the amount of neurotransmitter released

Reassuming the expression and functional consequences of the group III mGlu receptors family it can be concluded, that the receptors are present both on pyramidal and interneuronal terminals. The pyramidal neurons expressing mGlu4/7/8 receptors may contact with both the interneurons and pyramidal cells, but the GABAergic terminals expressing mGlu4, mGlu7 and mGlu8 receptors form the most synapses with the interneurons (predominantly mGlu1α-somatostatin positive), yet not with the pyramidal cells (Kogo et al., 2004), depressing the IPSCs of the inhibitory neuronal elements. Those receptors are activated with the glutamate released by the glia, or heterosynaptically by the glutamate released in the neighboring synapse. Such glutamate spillover enables the synapse to cooperate in regulating the excitatory/inhibitory balance in CNS.

8. Conclusions

The search for new anxiolytic therapy is one of the key areas of modern research, and a hope for the growing number of people affected by anxiety disorder. Besides, the anxiety is commonly comorbid with different psychiatric, and somatic, illnesses, so the proper treatment can constitute a supplementary therapy. There is no better way to improve pharmacological treatment than understanding the complex interaction between excitation/inhibition in the CNS. All that was written in these few pages until now states

that the receptors mediating both fast and slow excitatory or inhibitory currents are present at a broad range of synapses that are postulated to be critical for the maintenance of the correct balance in the brain. The complex interactions between synaptic responses, the releases of the neurotransmitters and receptor trafficking (not discussed here) at the excitatory glutamatergic or inhibitory GABAergic synapses is more complicated than anyone could ever imagine.

More than one type of auto- heteroreceptor can be expressed on one nerve terminal, so the receptors may cooperate with, or antagonize, each other's action. Such a cooperation has already been shown for mGlu7/$GABA_B$ receptors. Besides, each type of pyramidal neuron is likely to be innervated by multiple, functionally distinct GABA cells, which may differ in the mGlu expression. Some other factors, such as variation of the presynaptic receptor level in individual terminals or the state of activation or desensitization of the receptor, may also be important in the final effect of the treatment.

9. Acknowledgements

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9

Role of the Endocannabinoid System in Anxiety and Stress-Related Disorders

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1. Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder that may develop after exposure to a life-threatening traumatic experience and according to the DSM-IV diagnostic criteria, involves characteristic features such as persistent experiencing of trauma, avoidance, numbing, and hyperarousal (DSM-IV, 1994). Although many individuals with PTSD recover during the first couple of years following traumatic exposure, up to 30–40% remain chronically symptomatic (Kessler et al., 1995). Furthermore, individuals with chronic PTSD were shown to maintain conditioned fear responses to traumatic stimuli even 40–50 years after the trauma (Orr et al., 1993).

The largest body of treatment literature exists for antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs). Extensive clinical experience with these agents and their efficacy for disorders that are commonly comorbid with PTSD means that their use will likely continue until better treatment options are found (Davidson, 2000; Ravindran & Stein, 2009) even though their efficacy and that of other pharmacological treatments is generally limited. The high prevalence, chronicity, and resistance to treatment underscore the importance of the development of effective therapeutic strategies for PTSD.

The endocannabinoid (eCB) system has arisen as part of the complex circuitry that regulates anxiety and as a crucial mediator of emotional learning (Marsicano et al., 2002; Viveros et al., 2005; Laviolette & Grace, 2006a,2006b; Gorzalka et al., 2008; Ganon-Elazar & Akirav, 2009; Lutz, 2009; Hill & Gorzalka, 2009; Parolaro et al., 2010). The idea that the eCB system is involved in the control of anxiety-like behaviour is rooted in the fact that *Cannabis sativa* is used recreationally, mainly for its euphoric effects. However, this picture is confounded by the fact that cannabis abusers sometimes experience dysphoric reactions, with feelings of anxiety and panic. Additionally, it has been suggested that a temporary form of drug-induced psychosis can occur in some cannabis users. This 'marijuana psychosis' is usually due to exposure to large doses of the drug (Iversen, 2003) and has similar symptoms to paranoid schizophrenia. This has led some to propose a 'cannabinoid hypothesis of schizophrenia', suggesting that the symptoms of schizophrenic illness might be caused by an abnormal over-activity of endogenous cannabinoid mechanisms in the brain (Emrich et al., 1997).

An interesting question in cannabis research is whether cannabis use can precipitate long-term psychiatric illness. There are several reports suggesting that the development of cannabis dependence in young people is associated with increased rates of psychiatric symptoms, including psychosis, depression and anxiety (Andreasson et al., 1987; Mathers &

Godse, 1992; Hall & Degenhardt, 2000; Johns, 2001; Patton et al., 2002). In general, these studies do not prove any cause-and-effect relationship between cannabis use and long-term psychotic illness. Yet, cannabis can exacerbate the symptoms of existing psychotic illness. This of course will limit the therapeutic use of cannabis in people with existing psychotic illness (Iversen, 2003).

The psychoactive constituent of marijuana, delta-9-tetrahydrocannabinol (THC), was identified in 1964 (Gaoni & Mechoulam, 1964) and this discovery led to the identification of the endogenous eCB system. This system includes cannabinoid receptors (CB1 and CB2), eCBs (anandamide and 2-arachidonoyl-glycerol [2-AG]), enzymes involved in their synthesis and metabolism (fatty acid amide hydrolase (FAAH) for anandamide and the monoacylglycerol lipase (MAGL) for 2-AG), and an eCB transporter (Devane et al., 1992; Freund et al., 2003; Kogan & Mechoulam, 2006). Endocannabinoids are synthesized 'on demand' at post-synaptic sites of neurons after increase in neural activity and calcium ion influx, and are then released into the synaptic cleft. Their main function appears to be the suppression of neurotransmitter release from the presynapse. Thus, eCBs act as retrograde neurotransmitters, modulating other neurotransmitter systems.

Anandamide, 2-AG, FAAH, and the CB1 receptor are expressed in brain areas involved in stress, fear, emotions, and reward, including the amygdala, nucleus accumbens (NAc), hippocampus, and prefrontal cortex (PFC)(Herkenham et al., 1991; Freund et al., 2003; Pazos et al., 2005; Svizenska et al., 2008; Breivogel & Sim-Selley, 2009). Hence, the significant changes in mood and anxiety induced by cannabis use are in accordance with the presence of CB1 receptors in these brain regions. CB1 and CB2 receptors belong to the superfamily of G protein coupled receptors (Devane et al., 1988; Howlett et al., 1990), the CB1 receptor is widely distributed in the terminals of neurons (Herkenham et al., 1991), while the CB2 receptor is extensively expressed throughout the immune system (Piomelli , 2003). However, it has recently been reported that these receptors are present also in the brain (Gong et al., 2006; Onaivi et al., 2006).

In this review, I will provide pre-clinical support for the view that the eCB system may represent a therapeutic target for the treatment of stress-related diseases and others characterized by an inability to extinguish maladaptive behaviours, in particular PTSD. Studies of the eCB system support its importance for multiple aspects of brain function, including modulation of the hypothalamic-pituitary-adrenal (HPA) axis, regulation of the stress response, anxiety, and extinction of fear learning (Viveros et al., 2007; Taber & Hurley, 2009). I will give evidence that generally enhancing eCB signalling could represent a novel approach to the treatment of anxiety-related disorders whereas dysregulating the eCB system may result in anxiety and stress-related disorders. I will also focus on the role of the eCB system in brain circuits implicated in neuropsychiatric conditions, such as those modulating stress reactions, learning, extinction of fear, and emotional regulation (i.e., amygdala, NAc, hippocampus, and PFC) (Akirav & Maroun, 2007; Jankord & Herman, 2008).

2. Role of the endocannabinoid system in stress, anxiety, and fear

2.1 The neurocircuitry of stress, anxiety and fear

Stress is most readily defined as any stimulus that presents a challenge to homeostasis including any actual or potential disturbance of an individual's environment. The stress response enables the animal to adapt to the changing environment (Joëls & Baram, 2009).

Fear is an adaptive component of the acute stress response to potentially dangerous stimuli that threaten the integrity of the individual. However, when disproportionate in its intensity, chronic, irreversible and/or not associated with any actual risk, it constitutes a maladaptive response and may be symptomatic of anxiety-related neuropsychiatric disorders (Taber & Hurley, 2009).

Anxiety disorders are marked by excessive fear (and avoidance), often in response to specific objects or situations, in the absence of true danger, and they are common in the general population (Shin & Liberzon, 2010). As excessive fear is a key component of anxiety disorders, the search for the neurocircuitry of anxiety disorders has focused extensively on studies of fear circuits in animal models. These studies examined the neurocircuitry associated with fear responses in rats and mice using fear conditioning paradigms, inhibitory avoidance, and fear-potentiated startle models. The amygdala, PFC, and hippocampus have arisen as clear regions of interest in studies of anxiety disorders and are implicated in PTSD (Shin & Liberzon, 2010).

The hippocampus is often implicated in the neurobiology of stress. Mineralocorticoid and glucocorticoid receptors are expressed in high numbers within the hippocampus. Although stress-induced corticosteroid signalling in the hippocampus has a beneficial role in regulating the time course of the HPA axis stress response (de Kloet et al., 2005), prolonged glucocorticoid signalling can damage the hippocampus as measured by dendritic atrophy, decreased neurogenesis, and deficits in synaptic plasticity (McEwen & Gould, 1990; Sapolsky, 1996; McEwen, 1999; Meaney, 2001). In PTSD and major depression patients, hippocampus volumes are reduced (Bremner et al., 1995; Sheline et al., 1999; Woon & Hedges, 2008), and smaller hippocampal volumes are predictive of vulnerability to develop stress-related disorders (Pitman et al., 2006).

The amygdala plays a role in the control of emotional and autonomic responses to stress. It is involved in mood regulation and in the mediation of fear and anxiety (Davis, 1992; LeDoux, 2007) and is bi-directionally related to the frontal cortex (McDonald, 1998) and hippocampus (Pitkanen et al., 2000). Studies of fear conditioning, pharmacologically induced fear, and responses to emotional stimuli and facial expressions have provided evidence that the human amygdala, although responsive to multiple salient stimuli, responds reliably and potentially preferably to stimuli that predict threat and can be involved in mediating fear/anxiety states. Given that patients with anxiety disorders experience fear and distress in response to possible predictors of threat, the amygdala has been hypothesized to be hyperresponsive in some anxiety disorders. According to some models, the amygdala is hyperresponsive in PTSD, which may account for exaggerated fear responses and the persistence of traumatic memories (Shin and Liberzon, 2010).

The PFC plays an integral role in mediating a range of executive functions that subserve the selection and processing of information necessary to plan, control and direct behaviour in a manner appropriate to current environmental demands (Goldman-Rakic, 1996; Rolls, 1996; Tremblay & Schultz, 1999; Bush et al., 2000; Miller & Cohen, 2001; Robbins, 2005). A growing literature from studies in laboratory animals demonstrates that the PFC not only plays a major role in orchestrating the behavioural and systemic response to stress, but that neurons in the rodent PFC are highly sensitive to stress and undergo significant remodelling following stress exposure. These findings support the notion that stress-induced alterations in PFC function represent a principle neural insult underlying deficits in executive function

observed in stressed rodents, and the executive component of many neuropsychiatric diseases (for review see: Holmes & Wellman, 2008).

The amygdala and hippocampus have well established roles in encoding and processing memory for emotional or stressful events into long term storage (Cahill et al., 1996; Canli et al., 2000; Richter-Levin & Akirav, 2003; Joels et al., 2004; McGaugh, 2004; Phelps, 2004; Vianna et al., 2004; Diamond et al., 2005). However, stress effects on memory cannot be explained through only hippocampus and amygdala mediated alterations. There is growing interest in other brain areas, particularly the NAc, involved in mediating stress-related dysfunction (Willner et al., 1992; Zangen et al., 2001; Nestler et al., 2002). The NAc shell division may play an important role in integrating and consolidating representations of new experiences that are initially processed by both the amygdala and hippocampus (Seamans & Phillips, 1994; Setlow et al., 2000; Reynolds & Berridge, 2002). Anatomical findings demonstrate that the shell receives neural input from the BLA concerning the affective components of experiences (Mogenson et al., 1980; Petrovich et al., 1996), projections from the ventral subiculum region of the hippocampus regarding contextual features from the environment (Groenewegen et al., 1987; Meredith et al., 1990) and reward related components of learning experiences from the ventral tegmental area (Nauta et al., 1978).

2.2 Role of the endocannabinoid system in unconditioned stress and anxiety

Results from many studies indicate that the eCB system modulates unconditioned stress- and anxiety-like responses (Viveros et al., 2005; Gorzalka et al., 2008; Lutz, 2009). A general conclusion that can be tentatively derived from the complicated and often contradictory literature is that inhibition of eCB signalling increases stress and anxiety, while moderate increases in eCB signalling decrease stress and anxiety (Lutz, 2009). The term "moderate" is used because strong stimulation of eCB signalling by high doses of CB1 receptor agonists potentiate stress- and anxiety-like responses (Rodriguez de Fonseca et al., 1996; Scherma et al., 2008; Lutz, 2009). This biphasic effect has been demonstrated in animal models of anxiety (Lafenetre et al., 2007; Hill & Gorzalka, 2009), and also in humans. Cannabis may induce aversive states in some smokers, precipitating anxiety and panic attacks (Hall & Solowij, 1998). Furthermore, THC administration may result in psychotic-like states (Linszen & van Amelsvoort, 2007). These bidirectional effects of cannabinoids observed in humans can be mimicked in laboratory animals. Hence, in models predictive of anxiolytic-like activity, low doses of CB1 agonists tend to be anxiolytic and high doses tend to increase aversion and anxiety-related behaviours (Viveros et al., 2005).

Procedures used in studies on the role of eCBs in stress and anxiety evaluate the anxiolytic/anxiogenic effects of drugs by using standard tasks such as the elevated plus maze, social interaction, and defensive burying (Viveros et al., 2005; Lutz, 2009). Methods to modulate eCB signalling include genetic deletion of CB1 receptors, CB1 receptor antagonists (e.g., rimonobant/ SR141716, AM 251) and agonists (e.g., WIN55,212-2, HU210, which also activate CB2 receptors primarily found in the periphery (Freund et al., 2003; Di Marzo, 2008). Inhibitors of FAAH (URB597) or of the eCB transporter (AM404) have also been utilized, which leads to increased synaptic levels of anandamide and 2AG (Kathuria et al., 2003; Viveros et al., 2005; Bortolato et al., 2006).

Using the elevated plus maze, Patel & Hillard (2006) found that cannabinoid receptor agonists WIN 55212-2 (0.3-10 mg/kg) and CP 55,940 (0.001-0.3 mg/kg) increase the time mice spend on the open arms (i.e. elicit an anxiolytic response) only at low doses. At the

highest doses, both compounds alter overall locomotor activity. In contrast, THC (0.25-10 mg/kg) produces a dose-dependent reduction in time spent on open arms. The eCB uptake/catabolism inhibitor AM404 (0.3-10 mg/kg) produces an increase in time spent on the open arms at low doses and has no effect at the highest dose tested. The FAAH inhibitor URB597 (0.03-0.3 mg/kg) produces a monophasic, dose-dependent increase in time spent on the open arms. The CB1 receptor antagonists SR141716 (1-10 mg/kg) and AM251 (1-10 mg/kg) produce dose-related decreases in time spent on open arms. Onaivi et al., (1990) have shown that THC induces increased aversion to the open arms of the elevated plus maze in both rats and mice that is similar to the aversion produced by anxiogenic agents. In contrast, mice treated with the agonists cannabidiol and nabilone spend a greater amount of time in the open arms of the maze, an effect similar to that produced by diazepam, the reference anxiolytic agent.

In the light-dark box, Berrendero & Maldonado (2002) have shown that the administration of a low dose of THC (0.3 mg/kg) produces clear anxiolytic-like responses. The CB1 cannabinoid receptor antagonist, SR 141716A (0.5 mg/kg) completely blocks the anxiolytic-like response induced by THC, suggesting that this effect is mediated by CB1 cannabinoid receptors. In another study, systemic administration of the FAAH inhibitors URB597 and URB532 reduces anxiety-related behaviour in the rat elevated zero-maze and isolation-induced ultrasonic vocalisation tests (Kathuria et al., 2003). These effects are dose-dependent and blocked by the antagonist rimonabant. The FAAH inhibitor and eCB re-uptake inhibitor AM404 also exhibit a dose-dependent anxiolytic profile in the elevated plus-maze, defensive withdrawal test, and ultrasonic vocalisation test (Bortolato et al., 2006). URB597 has also been shown to be anxiolytic in the rat elevated plus-maze and open-field tests (Hill et al., 2007) and has recently been shown to reduce anxiety-related behaviour in the elevated plus-maze in Syrian hamsters (Moise et al., 2008).

Ribeiro et al. (2009) examined the dose-response effects of exogenous anandamide at doses of 0.01, 0.1, and 1.0 mg/kg in mice sequentially submitted to the open field and elevated plus-maze. Administered at 0.1 mg/kg (but not at 0.01 or 1 mg/kg), anandamide increases the time spent and the distance covered in the central zone of the open field, as well as exploration of the open arms of the elevated plus-maze. Recently, Rubino 2008b demonstrated that the anxiolytic-like effect of a low anandamide dose is reversed by administration of the antagonist AM251, whereas the anxiogenic-like effect is inhibited by pre-treatment with capsazepine, a transient receptor potential vanilloid type 1 (TRPV1) receptor antagonist. The authors suggested that the anxiolytic effect evoked by anandamide might be due to the interaction with the CB1 cannabinoid receptor, whereas vanilloid receptors seem to be involved in the anxiogenic action of anandamide (Rubino 2008b). Marsch et al. (2007) reported that TRPV1 "null" mice exhibit a significantly reduced response to anxiogenic stimuli. Therefore, the anandamide-induced inverted U-shape pattern might be based on the fact that the intrinsic efficacy of anandamide on TRPV1 is relatively low compared to that observed on the CB1 receptor (Ross, 2003).

Transgenic mice deficient for FAAH, the enzyme that degrades anandamide, demonstrate reduced anxiety-like behaviour in the elevated plus maze and light-dark box compared with wild-type mice and these effects are prevented by systemic administration of the antagonist rimonabant (Moreira et al., 2008). On the other hand, transgenic mice lacking expression of the CB1 receptor demonstrate an anxiogenic profile in the elevated plus-maze, the light-dark box, open-field arena, and social interaction test (Maccarrone et al., 2002; Martin et al., 2002; Haller et al., 2002; 2004; Uriguen et al., 2004) and demonstrate impaired stress coping behaviour in the forced swim test (Steiner et al., 2008). Similarly, CB1 receptor antagonists

increase anxiety-related behaviours in the elevated plus maze (Patel & Hillard, 2006). Taken together, these studies suggest that eCBs act at CB1 receptors to reduce anxiety.

2.3 Role of the endocannabinoid system in conditioned fear and anxiety

Understanding the role of the eCB system in conditioned fear and aversive memories is important because a number of anxiety disorders, including PTSD and phobias, are thought to result from dysregulated fear neurocircuitry (Rauch et al., 2006). Conditioned fear is induced by pairing a neutral, conditioned stimulus (CS; e.g., a light, a tone, or a context) with an aversive stimulus (unconditioned stimulus, US; e.g., a mild footshock) that evokes a measurable fear response. Evoked fear responses have been used extensively in animal models to better understand the mechanisms by which aversive memories are formed, and to model diseases such as PTSD and specific phobia, where inciting cues lead to the production of pathological fear states.

Investigators have examined the effect of CB1 receptor agonists and antagonists on contextual and cue fear conditioning. Results from these studies were somewhat mixed. In rats, systemic injections of the CB1 receptor antagonist AM251 enhance both the acquisition and expression of cue fear conditioning (Arenos et al., 2006; Reich et al., 2008). Administering AM251 (5 mg/kg, i.p) during tone–footshock conditioning enhances acquisition of freezing behaviour for both trace fear conditioning (hippocampal-dependent) and delay fear conditioning (amygdala–dependent) (Reich et al., 2008). Recently, we used an inhibitory avoidance task and found that microinjecting AM251 (6 ng) into the BLA significantly enhances conditioned avoidance but has no effect on conditioning when microinjected into the hippocampal CA1 area (Ganon-Elazar & Akirav, 2009; Abush & Akirav, 2010). However, others have shown that mice lacking the CB1 receptor or systemically administered with the CB1 receptor antagonist AM251 (0.3-3 mg/kg) 30 min before behavioural testing show no contextually induced fear response (Mikics et al., 2006) and that the CB1 receptor antagonist rimonobant or genetic deletion of the CB1 receptor has no effect on the acquisition of cue and context fear conditioning in mice (Marsicano et al., 2002; Suzuki et al., 2004). On the other hand, cue-fear-potentiated startle is decreased by medial PFC injections of the CB1 receptor agonist WIN55212-2 or the FAAH inhibitor URB597 (Lin et al., 2008, 2009) and contextual fear conditioning is decreased by dorsolateral periaqueductal gray injections of either anandamide or the anandamide transport inhibitor AM404 (Resstel et al., 2008). Overall it appears that, as in the case of unconditioned fear, inhibition of eCB transmission increases fear while moderate stimulation of eCB transmission decreases fear.

2.4 Role of the endocannabinoid system in extinction

Extinction was established as a tool to treat conditioned fear by Freud in the 1920s. It has become widely accepted that a deficit in the capacity to extinguish memories of fear is at the root of fear disorders as a result of the distinction between those who do and do not develop serious symptoms after fearsome experiences, and the fact that fear disorders are treated with therapy based on extinction procedures. Moreover, panic attacks, phobias, and particularly PTSD are viewed by many as a deficit of extinction that should therefore be treated by an intensification of extinction (Charney et al., 1993; Wessa & Flor, 2007; Milad et al., 2008).

Experimental extinction learning occurs when a CS that previously predicted a US no longer does so, and over time, the conditioned response (e.g., freezing or elevated skin conductance responses) decreases. Extinction learning involves the ventromedial PFC, amygdala and hippocampus (Milad and Quirk, 2002; Phelps et al., 2004; Bouton et al., 2006). PTSD patients exhibit long-lasting reexperience of traumatic events and avoidance of the trauma-related stimuli, even though they recognize that the traumatic event is no longer occurring. It has been suggested that dysfunctional fear extinction plays an important role in the development of clinical symptoms, such as reexperiencing of trauma, in PTSD (Rothbaum et al., 2003; Quirk et al., 2006; Rauch et al., 2006; Milad et al., 2006). Furthermore, in a recent study, PTSD patients demonstrated deficient extinction recall as measured in skin conductance response in a 2-day fear conditioning and extinction procedure (Milad et al., 2008).

Clearly, animal models do not entirely mimic the complex features of psychiatric disorders. However, they can predict the clinical effects of substances and provide insights into the biological mechanisms of these diseases. Marsicano et al. (2002) found that CB1 receptor-deficient mice show normal acquisition and consolidation in a fear conditioning task, but fear extinction is strongly impaired. Impaired extinction is also observed when the antagonist SR141716 is injected systemically into wild-type mice before the extinction trial, indicating that CB1 receptors are required at the moment of the extinction training. The findings that CB1 knockout mice exhibit impaired short- and long-term extinction of cue-induced conditioned fear responses have been replicated by other groups both for extinction of cue- or context-induced fear responses (Suzuki et al., 2004; Finn et al., 2004; Chhatwal et al., 2005; Lafenetre et al., 2007; Lutz, 2007; Niyuhire et al., 2007). We have recently shown that microinjecting the antagonist AM251 (6 ng) into the BLA or the CA1 significantly impairs extinction of inhibitory avoidance (Ganon-Elazar & Akirav, 2009; Abush & Akirav, 2010). Regarding the extinction of non-aversive memories, several studies suggested that the eCB system is not involved (Hölter et al., 2005; Niyuhire et al., 2007).

On the other hand, studies have demonstrated that pharmacological activation of eCB signalling promotes extinction of fear memories. For example, Chhatwal et al. (2005) found that systemic administration of AM404 (10 mg/kg) promotes extinction of conditioned fear using fear potentiated startle. This was replicated using systemic (Pamplona et al., 2008) and intracerebroventricular (Bitencourt et al., 2008) injections. In another study (Varvel et al., 2007), OL-135 (30 mg/kg), an inhibitor of FAAH, enhanced the rate of extinction in a water maze task. Pamplona et al. (2006) showed that WIN 55-212,2 (0.25 mg/kg) facilitates the extinction of contextual fear in the fear conditioning task and of spatial memory in the water maze reversal task. We demonstrated that WIN 55,212-2 administered into the CA1 facilitates the extinction of inhibitory avoidance, with no effect on extinction kinetics when microinjected into the BLA (Ganon-Elazar & Akirav, 2009; Abush & Akirav, 2010).

Results of Marsicano et al. (2002) and subsequent investigations demonstrate that inhibition of eCB transmission robustly inhibits (or prolongs) fear extinction (Suzuki et al., 2004; Pamplona et al., 2006). Conversely, stimulation of eCB transmission accelerates fear extinction (Suzuki et al., 2004; Chhatwal et al., 2005; Barad et al., 2006; Abush & Akirav, 2010). However, the role of eCB in the extinction of aversive memories is to some degree task-specific and negative results were reported (Kobilo et al., 2007; Lutz, 2007).

There is evidence to suggest that cannabinoids act on anxiety responses and fear learning through their effects on the amygdala. Central CB1 receptors are expressed at high levels in

the lateral and basal nuclei of the amygdala (Katona et al., 2001), and extinction of aversive memories depends on cannabinoid receptors and signalling within the BLA (Marsicano et al., 2002; Kamprath et al., 2006;Laviolette and Grace 2006a). It has been demonstrated that activation of CB1 receptors attenuates anxiety responses and amygdala activation to aversive stimuli by modulating neuronal firing in the BLA (Pistis et al., 2004; Patel et al., 2005). We have demonstrated (Ganon-Elazar & Akirav, 2009) that exposure to acute stress enhances conditioned avoidance and impairs inhibitory avoidance extinction. Intra-BLA WIN55,212-2 (5 μg) prevents the stress-induced enhancement of conditioned avoidance as well as the stress-induced disruption of avoidance extinction (see **Figure 1**). This reversal effect was found to be associated with alterations in the HPA axis, as intra-BLA WIN55,212-2 inhibits the stress-induced increase in plasma corticosterone levels (Ganon-Elazar and Akirav, 2009; see **Figure 2**). These findings support a possible therapeutic application for cannabinoids in the treatment of conditions associated with stress-related disorders and the inappropriate retention of aversive memories.

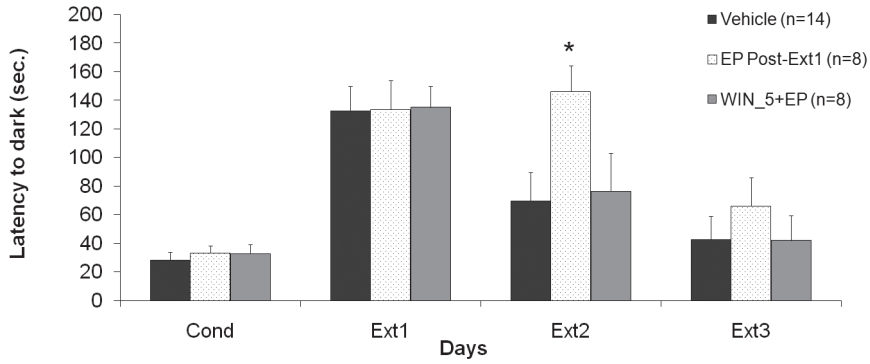

Fig. 1. Cannabinoid receptor agonist WIN55,212-2 (5 μg/0.5 μl) blocks the effects of stress on inhibitory avoidance extinction

After the first extinction trial (Ext1), rats were microinjected with vehicle (n = 14), placed on the elevated platform (EP Post-Ext1, n = 8) stress, or microinjected with WIN55,212-2 into the BLA and immediately afterward placed on the EP (WIN_5+EP, n = 8). The EP Post-Ext1 group showed a significantly increased latency to enter the dark side on the second extinction day compared with the other groups (Ext2, $p < 0.05$). Thus, WIN55,212-2 administered into the BLA before stressor exposure prevents the disrupting effect of the stressor on IA extinction (data published by Ganon-Elazar and Akirav, 2009 in J Neurosci).

2.5 Endocannabinoid system modulation of the hypothalamic-pituitary-adrenal axis (HPA) axis

A body of evidence has emerged indicating a key role for the eCB system both in regulating basal HPA axis activity and in 'fine-tuning' the HPA axis response to stress (Finn, 2010). However, it should be borne in mind that elevation of eCB levels sometimes has effects that are different from those observed with exogenous cannabinoids, especially regarding eCB modulation of the HPA axis.

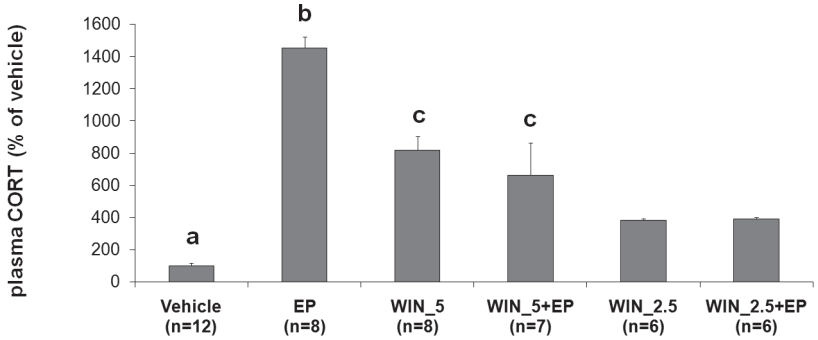

Fig. 2. The effects of the cannabinoid receptor agonist WIN55,212-2 and stress on corticosterone levels

Corticosterone levels were measured in rats microinjected with vehicle into the BLA (vehicle, n = 12), placed on the EP (n = 8), microinjected with WIN55,212-2 into the BLA (5 µg; WIN_5, n = 8), microinjected with WIN55,212-2 into the BLA and placed on the EP (5 µg; WIN_5+EP, n = 7), microinjected with a lower dose of WIN55,212-2 into the BLA (2.5 µg; WIN_2.5, n = 6), or microinjected with the lower dose of WIN55,212-2 and placed on the EP (WIN_2.5+EP, n = 6). Data represent the means ± SEM expressed as a percentage of the corticosterone values of the vehicle animals (corticosterone levels in the vehicle group, 95.52 ± 16.7 ng/ml) ($p<0.05$, a: Vehicle group differs from all other groups; b: EP group differs from all other groups; c, differs from WIN_2.5 and WIN_2.5+EP groups) (data published by Ganon-Elazar and Akirav, 2009 in J Neurosci).

The HPA axis is the principal neuroendocrine component of the response to stress; during acute stress, corticotrophin releasing factor (CRF) is released into the portal system by neurons in the paraventricular nucleus (PVN). CRF induces release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH stimulates production of glucocorticoids (e.g., corticosterone in rats) in the adrenal gland. Glucocorticoids produce a range of effects on the cardiovascular, immune, metabolic, and neural systems that facilitate optimal responses to aversive stimuli (Pecoraro et al., 2006) but also exert potent negative feedback inhibition of the HPA axis glucocorticoids by inhibiting the release of CRF in the hypothalamus and ACTH in the pituitary. Hence, elevated circulating levels of glucocorticoids rapidly suppress HPA axis activity. Hypothalamic and extrahypothalamic limbic structures also regulate the HPA axis. The amygdala activates the HPA axis in response to stressful stimuli whereas the hippocampus and prefrontal cortex inhibit the HPA axis.

The relationship between stress and the eCB system is complex, and it has been reported that stressful events increase eCB levels in several brain areas, likely in response to the stimulation of glucocorticoid receptors (Di et al., 2005; Malcher-Lopes et al., 2006). Stress elicits the rapid formation of eCBs in the periaqueductal gray matter of the midbrain (Hohmann et al., 2005) and alters eCB content in the limbic forebrain, amygdala, striatum, and PFC Patel et al., 2005; Rademacher et al., 2008). Smoking marijuana in humans or administration of THC in animals stimulates ACTH and glucocorticoid secretion (Murphy et al., 1998; Manzanares et al., 1999). We found that intra-BLA administration of WIN 55,212-2 increases corticosterone levels in naïve rats, but is able to reduce the stress-induced elevation in corticosterone levels (Ganon-Elazar & Akirav, 2009). Similarly, Patel et al. (2004)

have shown that mice treated systemically with the eCB transport inhibitor AM404 or the FAAH inhibitor URB597 show significantly decreased or eliminated restraint-induced corticosterone release. Studies have shown a decrease in the content of the endogenous cannabinoid ligand anandamide in the amygdala, through an increase in FAAH-mediated hydrolysis, in response to acute stress (Patel et al., 2005; Rademacher et al., 2008; Hill et al., 2009). Preventing this decline in amygdalar anandamide by local administration of a FAAH inhibitor into the BLA attenuates stress-induced activation of the HPA axis (Hill et al., 2009). Hence, the augmentation of eCB signalling can suppress stress-responsive systems (Patel et al., 2004; Cota, 2008; Steiner & Wotjak, 2008). A low dose of CP55940 (0.03 mg/kg) suppresses stress-induced corticosterone release, whereas a high dose (0.3 mg/kg) facilitates corticosterone release in response to restraint, consistent with previous data that direct CB1 agonists have biphasic effects on HPA axis activation and anxiety-like behaviours. On the other hand, the absence of CB1 receptors in mice or the systemic administration of CB1 receptor antagonists results in enhanced stress-induced secretion of ACTH and corticosterone compared with wild-type controls (Manzanares et al., 1999; Barna et al., 2004; Haller et al., 2004; Patel et al., 2004; Steiner et al., 2008; Steiner & Wotjak, 2008). For example, acute systemic administration of the antagonists SR141716 or AM251 to rodents results in increased circulating corticosterone levels (Patel et al., 2004; Wade et al., 2006; Steiner et al., 2008; Ganon-Elazar and Akirav, 2009). Basal plasma ACTH concentrations in response to novelty stress are increased in CB1 knock-out mice compared with wild-type animals (Haller et al., 2004), hence suggesting that eCB signalling negatively modulates HPA axis activity.

Taken together these findings demonstrate that the eCB system is an important regulator of the central stress response and that alterations of the eCB tone might be helpful in reducing the stress response and may be effective in preventing the occurrence of stress-related diseases.

There are several mechanisms by which cannabinoids may exert their anxiolytic effects via stress-related hormonal systems. The BLA is thought to process aversive sensory stimuli via afferent inputs to the central amygdala (CeA; LeDoux, 2000). It is also believed that GABAergic neurons in the intercalated nuclei serve as an intermediate relay station to generate feed forward inhibition of CeA after activation by BLA (Pare & Smith, 1993). The effects of selective CB1 agonists on GABA-mediated inhibitory postsynaptic currents at lateral, but not central, amygdala nuclei may reduce inhibitory tone on BLA cells. Thus, CB1 agonists reduce GABA release in BLA interneurons, thereby reducing their inhibition of the GABAergic neurons of the intercalated nuclei, which, in turn, increases their inhibition of the pyramidal neurons of the CeA (Katona et al., 2001). Hence, the reduction in inhibitory tone may in turn indirectly reduce anxiety by enhancing the activity of intercalated GABAergic cells that inhibit activation of the CeA (Katona et al., 2001). In support, it has been shown that CB1 agonists decrease the excitability of projection neurons in the rat BLA (Pistis et al., 2004). In humans, the benzodiazepine, pro-GABAergic anxiolytic agent lorazepam has been shown to attenuate amygdala reactivity to threatening faces (Paulus et al., 2005). Another possibility is that cannabinoids decrease CRH levels in the CeA, and decreased CRH levels are associated with decreased aversive stress responses (Rodriguez de Fonseca et al., 1997). Clearly, CB1 receptor activation may also involve other neurochemical (serotonin, cholecystokinin, opioid, etc.) systems relevant to anxiety and fear behaviours (Viveros et al., 2005).

2.6 The endocannabinoid system in a brain circuit involved in stress and anxiety

The eCB system participates in multiple brain circuits implicated in neuropsychiatric conditions, such as those modulating stress reactions, learning, extinction of fear, emotional regulation, and reward processes (e.g., the amygdala, hippocampus, NAc, and PFC). Neuroimaging studies have revealed that these structures are indeed active in individuals who smoked cannabis (Chang & Chronicle, 2007). This notion is further supported by experiments detecting molecular correlates of neural activity in cannabinoid-treated laboratory animals. For example, THC causes c-fos expression in the amygdala and the NAc (McGregor et al., 1998) and a significant increase in cAMP response element-binding (CREB) activation in the PFC and hippocampus (Rubino et al., 2007).

Techniques based on intracranial injections of cannabinoids in rats revealed that activation of CB1 receptors, specifically in some of the structures mentioned above, is involved in inducing anxiolytic- or antidepressant-like effects (Bambico et al., 2007; Moreira et al., 2007; Rubino et al., 2008a, 2008b). For example, Rubino et al. (2008a) found that low doses of THC microinjected into the PFC (10 μg) or ventral hippocampus (5 μg) in rats induces an anxiolytic-like response during tests in the elevated plus-maze, while higher doses do not show an anxiolytic effect and even seem to switch into an anxiogenic profile. Similarly, low doses of the anandamide analogue, methanandamide (0.1 μg), microinjected into the PFC, produce an anxiolytic-like response in rats, whereas higher doses (10 μg) induce anxiety-like behaviours, as indicated by the number of entries onto and percentage of time spent on the open arm of a plus maze (Rubino et al., 2008b). Yet, other studies demonstrated that eCB activation in the amygdala and dorsal hippocampus results in an anxiogenic-like response. Low THC doses (1 μg) in the BLA produce an anxiogenic-like response whereas higher doses are ineffective (Rubino et al., 2008a). WIN-55212-2 in the dorsal hippocampus (2.5 and 5 μg) produces a significant anxiogenic-like effect in rats that is reversed by AM251 (Roohbakhsh et al., 2007).

Local infusion of cannabinoid compounds into specific brain areas might be instrumental to identify neural pathways and neuroanatomically separated CB1 receptor subpopulations that may play distinct roles in and mediate the opposing actions of cannabinoids, notably, anxiolytic versus anxiogenic effects (Moreira et al., 2007; Viveros et al., 2007).

Although considerable evidence suggests that activation of CB1 receptors can induce learning and memory impairments (Sullivan, 2000; Robinson et al., 2003; O'Shea et al., 2004; Varvel et al., 2005), CB1 receptors are essential for the extinction of conditioned fear associations (Marsicano et al., 2002), indicating an important role for this receptor in neuronal emotional learning and memory. Laviolette and Grace 2006a demonstrated that CB1 receptors within the BLA-PFC circuit can potently modulate the magnitude of emotional associative learning processes during both the acquisition and expression of learned, emotionally salient conditioned associations. Using in vivo single-unit recordings in rats, they showed that the agonist WIN 55,212-2 potentiates the response of medial PFC neurons to olfactory cues paired previously with a footshock, whereas this associative responding is prevented by the antagonist AM251. In an olfactory fear-conditioning procedure, WIN 55,212-2 microinjected into the medial PFC enables behavioural responses to olfactory cues paired with a normally subthreshold footshock, whereas AM251 completely blocks emotional learning.

Treatment with cannabinoids significantly increases dopamine release in the NAc (Chen et al., 1990; Tanda et al., 1997). Yet, in situ hybridization and immunocytochemical studies reported a low-level, or even lack of CB1 receptors in the NAc, whereas other brain regions,

such as the PFC, the hippocampus, and the amygdala, which densely innervate the NAc, show moderate to very high CB1 receptor levels (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Tsou et al., 1998; Egertova and Elphick, 2000). It has been suggested that the BLA, hippocampus and PFC are likely candidates for conveying the indirect effects of cannabinoids on dopamine release within the NAc, thereby contributing to reward processes (Katona et al., 2001).

To summarize the role of the eCB system in stress, anxiety, and conditioned fear, there is a general consensus that the effects of cannabinoid agonists on anxiety seem to be biphasic, with low doses being anxiolytic and high doses being ineffective or possibly anxiogenic. There are several important characteristics of the eCB system that might explain these different effects of eCB modulation. First, in a physiological situation, eCB synthesis, and thus CB1 receptor activation, occurs in particular activated neuronal circuits. This is a notable difference from the situation following pharmacological treatment with receptor agonists, when the agent activates all CB1 receptors in the brain regardless of their specific involvement in a particular physiological process. Second, the CB1 receptor is expressed in diverse brain structures of relevance to psychiatric disorders and is mainly located presynaptically where it can suppress the release of other neurotransmitters (Marsicano & Lutz, 1999; Mackie et al., 2005; Marsicano & Lutz, 2006). These neurotransmitters include the main inhibitory neurotransmitter GABA, the main excitatory neurotransmitter glutamate, as well as acetylcholine, noradrenaline, and serotonin (Katona et al., 1999; Harkany et al., 2005; Monory et al., 2006; Oropeza et al., 2007; Häring et al., 2007). Thus, synthetic compounds delivered systemically lack both the spatial and temporal specificity of endogenous compounds (Viveros et al., 2007; Lafenetre et al., 2007; Moreira & Lutz, 2008). This may explain not only the bell-shaped relationship between dose and effect that some studies have observed, but also why elevation of eCB levels sometimes has effects that are different from those observed with exogenous cannabinoids. Finally, the diversity of eCB ligands with their multiple synthetic and degradation pathways adds a further level of complexity to the eCB system (Di Marzo, 2008).

3. The endocannabinoid system in human studies

Cannabis can promote a relaxing and euphoric effect, thus relieving anxious states, or anxiety and panic attacks, depending on subjects and on the emotional state prior to use (Iversen, 2003). Other factors that affect the change in mood and anxiety-related responses are individual differences in absorption, the method of smoking, drug dose, previous history, anxiety level, and environmental context (Gonzalez, 2007; Moreira & Lutz, 2008; Taber & Hurley, 2009). Whilst variable, the effects of low doses are often described as rewarding, producing feelings of a "high", relaxation, reduced anxiety, and increased sociability (Hall & Solowij, 1998; Murray et al., 2007). Human recreational cannabis users often report that low doses of the drug produce feelings of calmness and decreased anxiety (Abood and Martin, 1992; Porter and Felder, 2001). But high doses of THC modulate subjective anxiety (D'Souza et al., 2004). In any case, adverse reactions do occur (e.g., anxiety, panic, paranoia, psychotic symptoms), but are much less common than positive effects (Iversen, 2003; Kalant, 2004; Gonzalez, 2007). Functional imaging studies indicate that intoxication is associated with increased regional cerebral blood flow and metabolism, particularly in the frontal and limbic regions as well as the cerebellum (Gonzalez, 2007).

Some studies support an influence of cannabis use on the development of psychiatric disorders, particularly schizophrenia and mood disorders (Iversen, 2003; Kalant, 2004; Leweke & Koethe, 2008).

There is thus evidence that cannabis can have both anxiogenic and anxiolytic effects. These apparently conflicting observations may partly reflect the fact that *Cannabis sativa* contains multiple compounds that may have different psychoactive properties (Ashton, 2001). A recent study by Fusar-Poli et al., (2009) used functional magnetic resonance imaging (fMRI) to investigate the effects of the two main psychoactive constituents of *Cannabis sativa*, THC and cannabidiol, on the neural substrate of emotional processing. Cannabidiol and THC have distinct modulatory effects on the regional neural response to fearful faces. Cannabidiol attenuates the neurofunctional engagement of the amygdala and cingulate cortex when subjects view intensely fearful stimuli and this effect is correlated with a reduction in the electrodermal response, consistent with behavioural evidence that it has anxiolytic effects. In contrast, THC modulates activation in frontal and parietal areas and is associated with an increase in anxiety and the electrodermal response.

It is paradoxical that while individuals report reduced anxiety as the motivation for using cannabis, acute anxiety is the most common adverse effect of cannabis use, particularly at high doses (Crippa et al., 2009). Generally, acute anxiety following cannabis use is more common in drug-naïve subjects and when the drug is taken in novel or stressful environments. Cannabis use alone does not appear to be sufficient or necessary for the development of long-term anxiety but may be a risk factor that operates in conjunction with other vulnerability factors. Such factors include biological, neurodevelopmental, environmental and social influences, as well as personality traits or a combination of all of them (Lynskey et al., 2002; Windle & Wiesner, 2004; Chabrol et al., 2005).

Although not first-choice medications, generally cannabinoids are well tolerated after oral administration (Moreira et al., 2009). Some aversive effects that may result from cannabis smoking, such as anxiety and panic, are rarely observed after oral administration of the agonists nabilone and THC (Martyn et al., 1995; Berlach et al., 2006; Skrabek et al., 2008). An fMRI study examined 16 healthy, recreational cannabis users after a double-blind crossover oral administration of THC or placebo. They found that THC significantly reduces amygdala reactivity to social signals of threat but does not affect activity in the primary visual and motor cortex (Phan et al., 2008). Moreover, it has been recently shown that the synthetic cannabinoid nabilone has beneficial effects in PTSD patients in regard to abolishing or greatly reducing nightmares that persisted in spite of treatment with conventional PTSD medications (Fraser, 2009). Hence, oral administration may help avoid the high peak serum concentration that occurs after cannabis smoking or intravenous administration (Moreira et al., 2009).

4. Conclusions and future research directions

The involvement of the eCB system in multiple aspects of brain function provides new targets for the development of novel therapeutic agents for a wide range of psychiatric disorders, including the treatment of anxiety disorders.

A major problem with using the eCB system as a potential therapeutic target is the fact that it has a complex role in mediating anxiety states. In essence, there is a problem of a narrow therapeutic window between the efficacy against selected symptoms that are of clinical significance and the unwanted risks. Further understanding the functioning of the eCB

system should provide new therapeutic avenues that may avoid these psychiatric side-effects. For example, in addition to receptor agonists, a number of compounds have been developed that prolong eCB action, either by inhibiting uptake or by decreasing hydrolysis (Grant & Cahn, 2005; Pertwee, 2008). In particular, the specific FAAH inhibitor URB597 has anxiolytic-like properties without the sedative effects seen with cannabinoids, which directly activate CB1 receptors (Cota, 2008; Piomelli, 2008). Prolonging the activity of released anandamide using selective inhibitors of FAAH reduces anxiety indicating that this may be a therapeutically useful goal in the treatment of anxiety. Hence, modulation of neuronal endogenous cannabinoid signalling systems could represent a novel approach to the treatment of anxiety-related disorders while minimizing the adverse effects of direct action on cannabinoid receptor agonists.

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10

New Anxiolytic Phytopharmaceutical Elaborated with the Standardized Extract of *Galphimia glauca*

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1. Introduction

From ancient times, medicinal plants have played an important role in the treatment of diseases, especially in those related with the central nervous system (CNS). The study of opium properties (*Papaver somniferum*), marijuana (*Cannabis sativa* L.), mandragora (*Mandragora officinarum* L.), ayahuasca (*Banisteriopsis caapi* Spruce ex Griseb.), and peyote (*Lophophora williamsii* (Lem.) Coult.), among others, experienced an important boom during the 1960s and 1970s (Shultes & Hofmann, 2008). This research work was oriented toward discovering and demonstrating certain pharmacological properties in these plant species. However, the fascinating success of these investigations was the Identification of very interesting chemical compounds that were useful as pharmacological tools for the study of the CNS (Tortoriello, 1999). From plant species, it has been possible to obtain chemical compounds that have been the basis of the development of chemical pharmaceuticals, such as reserpine, morphine, atropine, caffeine, and physostigmine (McClatchey et al., 2009). Over the past several years, in some countries and continuing to refer to Western medicine, phytopharmaceuticals products (containing plant extracts) have been developed. Phytopharmaceuticals, recognized in some countries as officially accepted drugs, are products in which identification of the active compounds has been reached and in which effectiveness, tolerability, and safety have been demonstrated by means of controlled clinical trials (Tortoriello et al., 2003). The following phytopharmaceuticals with CNS properties have been used broadly worldwide: *Valeriana officinalis*, prescribed principally for the treatment of insomnia (Salter & Brownie, 2010); *Hypericum perforatum*, the extract obtained from its roots is used for producing phytopharmaceuticals useful for treatment of mild to moderate depression (Kasper et al., 2010), and *Ginkgo biloba*, a widely recognized plant species due to its properties of improving cerebral blood flow and because of its effect on

memory disorders (Mashayekh et al., 2010). Regarding anxiety, it has not been easy to discover plant species with demonstrated selective activity without the evidencing of side effects. *Piper methysticum* G. Forster (Kava Kava) is a plant originally from Oceania (Polynesia, Micronesia, and Melanesia) from which a very important anxiolytic phytopharmaceutical was developed. Despite that the pharmacological mechanism of action has not been identified, pharmaceutical products elaborated from *P. methysticum* root extract were evaluated in clinical trials in which an anxiolytic effect, different from that produced by benzodiazepines, was demonstrated (Gastpar & Klimm, 2003). Different double-blind clinical studies compared the effects produced by *P. methysticum* against placebo in patients with a diagnosis of nonpsychogenic anxiety and in women with menopause-associated anxiety. The phytopharmaceuticals produced with this plant extract achieved wide commercial success in Europe and in some countries on the American Continent. However, due to reports of some cases of hepatotoxicity, pharmaceutical products elaborated with this extract were withdrawn from the market in different countries. Recent studies, performed after the warning report, have continued to evaluate this product therapeutically (vs. buspirone and opipramol), but several studies have had the purpose of evaluating the toxicological effects (Teschke et al., 2011). At present, *P. methysticum* is out of the formal ethics pharmaceutical market in the majority of countries.

2. Ethnomedical use of *Galphimia glauca* in Mexican Traditional Medicine

Galphimia glauca Cav., of the Malpighiaceae family, is a medicinal plant native to Mexico that is commonly known with the name of "Calderona amarilla" (**Figure 1**). Although there are no written documents, it has been known that from past times, this plant species has been employed in Mexican Traditional Medicine as a sedative and a tranquilizer for persons with insanity. The tranquilizing properties of this plant were also utilized during the "Cristeros" War (1926-1929) in Mexico; soldiers who had profuse diarrhea, severe paleness, and fear of going to battle received an infusion prepared with the leaves and stems of this plant, which afforded them great tranquility and the disappearance of nervous diarrhea.
Galphimia glauca is a shrub that is widely distributed throughout Mexico due to its resistance to environmental conditions. Native to central Mexico, this evergreen plant has been known since pre-Columbian times. The Nahuatl people used the "totoncapatly" voice when referring to this shrub. Such an expression derives from the words "totonqui" and "patli", which mean "hot" and "medicine", respectively, in Nahuatl (Tortoriello, 1998).

3. Preliminary pharmacological studies of *G. glauca* extracts on the CNS

Based on the traditional medical use of this species, the methanolic extract of the aerial parts of the plant was evaluated in five neuropharmacological animal models. In all of these, the results obtained suggested a CNS depressor effect. The extract significantly potentiated the hypnotic effect induced by sodium pentobarbital in mice and reduced the effect of stimulant drugs administered in mice, such as strychnine and leptazol, producing in the animals an increment in the latency time of convulsions and diminishing the number of animals with convulsions, as well as mortality. Another depressor action was observed on the body temperature of the animals; administration in normothermic rats lowered the body temperature in a highly significant manner. On the other hand, it was demonstrated that *G. glauca* also depressed neuronal groups in *in vitro* isolated Guinea-pig ileum (Tortoriello & Lozoya, 1992).

Fig. 1. *Galphimia glauca* Cav. growing in a controlled crop.

4. Chemical isolation and identification of the active compound

By means of bioguided phytochemical separation, isolation was achieved of an active molecule whose structural elucidation demonstrated a nor-seco-triterpene. This compound is made up of 30 carbons organized in four rings with six members each, and a seventh member, hetero-cycle ring. Structurally, this compound comprises (4*R*)-Trihydroxy-13α-methoxycarbonyl-30-nor-3,4-seco-7α,18β-fridela-1,20-dien-3,24-olide, a new compound that received the trivial name of Galphimine-B or G-B (Figure 2). Chemical characterization was reached through exhaustive nuclear magnetic resonance (NMR) spectroscopic analysis of ^{1}H and ^{13}C, as well as x-ray diffraction of the crystallized compound (Toscano et al., 1993).
Other compounds, with similar structures, have been isolated from the active extract. These compounds also present the six-ring structure with the seventh hetero-cycle member, but with different functional groups (Figure 2). All of these, known as Galphimines, have shown to possess less activity than G-B (González-Cortázar et al., 2005).

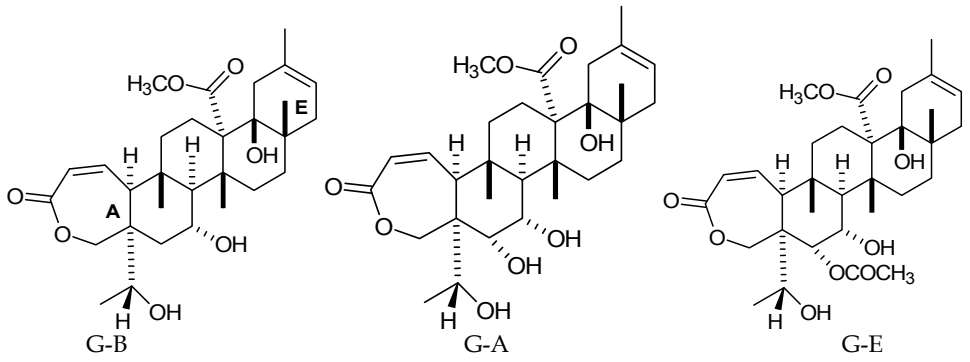

Fig. 2. Chemical structure of Galphimine- B (G-B), Galphimine-A (G-A), and Galphimine-E (G-E).

5. Anxiolytic effect and mechanism of action

5.1 Anxiolytic effect of metanolic extract from *Galphimia glauca*

In an assay carried out in ICR-strain male mice, it was demonstrated that the administration of increasing doses of the methanolic extract of *Galphimia glauca* (125, 250, 500, 1,000, and 2,000 mg/kg via oral route) caused a significant increase in animals in terms of the percentage of time that the mice remained on the open arms of the elevated plus maze (EPM), as well as also an increase in the percentage of number of crossings that the mice carried out toward these arms (p <0.05) (Figure 3). The data were compared with the group that only received a Tween 20 solution at 5% (vehicle, 10 µl/10 g of the weight of the mouse). The *G. glauca* extract utilized in this model was standardized in its G-B content, establishing that it contained 8.3 mg G-B/g of extract. As described in the literature (Rex et al., 2002), diazepam at 1.0 mg/kg was capable of inducing a significant increase in comparison with the control group (p <0.05) in the parameters previously cited, this indicative of pharmacological model validation (Herrera-Ruiz et al., 2006A).

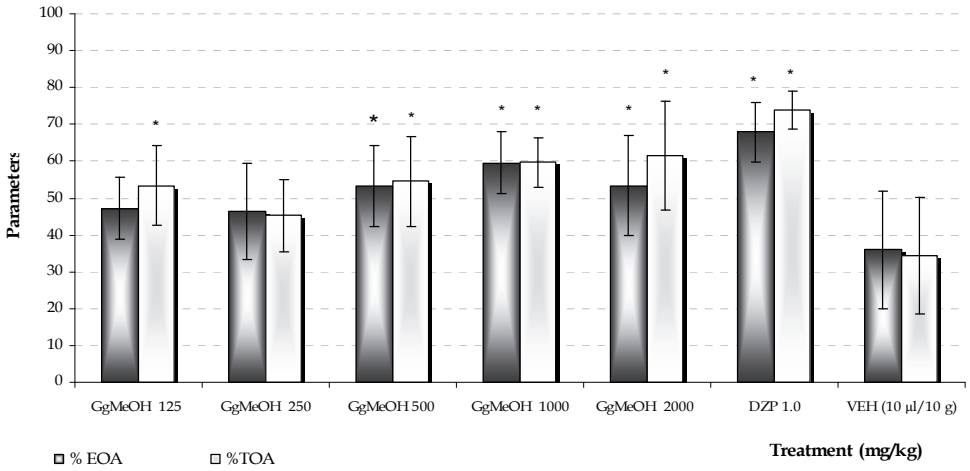

Fig. 3. Effect produced by different doses of methanolic extract from *Galphimia glauca* on ICR mice exposed to the elevated plus maze paradigm. *P <0.05 with ANOVA followed by post-hoc Dunnett test (mean ± Standard deviation [SD]; n = 7). DZP = diazepam; VEH = Vehicle (solution of Tween 20 at 5%). %EOA = mean percentage of the number entries on the open arms of the EPM; % TOA = mean percentage of time spent on the open arms of the EPM.

It has been proposed that adequate interpretation of the results in the EPM should be accompanied by an analysis of the ethological parameters that improve the model's sensitivity and that allow for identification of the emotional changes in the animals (Cole & Rodgers, 1994). These parameters can be confounder factors predicting the anxiolytic capacity of the treatments. In this way, it is suggested that within the EPM, it would be necessary to analyze the ethological measurements of Head dips (HD) (Weiss et al., 1998). Thus, for example, the increase in the number of HD on open arms is a behavior that is inversely correlated with the level of anxiety. This means that the more HD performed by the animals, the less anxiety demonstrated; this is a risk appraisal parameter, and this behavior is reported for substances such as diazepam (Cruz et al., 1994; Rodgers & Johnson,

1995). In the case of the administration of the methanolic extract of *G. glauca*, the number of HD was significantly greater (p <0.05) than that of the group that only received the vehicle; this parameter increased in a dose-dependent manner (Table 1). On the other hand, similar behavior was observed in the group of animals that received benzodiazepine .
Vertical exploration, or Rearings (R) is commonly accepted as a measurement of locomotor activity (Ramos et al., 1997). A decrease in the number of R can be related with the reduction in crossings to closed arms, where this behavior regularly occurs. It has been observed that diazepam causes a diminution in mice of the number of R, thus associating this with a diminution in the number of entrances on the closed arms. This behavior is in agreement with the results obtained with the increasing administration of the standardized *G. glauca* extract and also with diazepam; both treatments induce a significant diminution of R with respect to the vehicle (p <0.05) (Table 1).
In addition to the effect observed in the EPM model, we also evaluated the standardized extract of *G. glauca* (different doses) on the Light/dark model; this is a paradigm based on the rejection of rodents to travel through the brilliantly illuminated areas and on their spontaneous exploratory behavior in the face of the novelty of the environment (Crawley & Goodwing, 1980). It has been determined that this test is useful in predicting the anxiolytic or anxiogenic capacity of different treatments. Benzodiazepines and serotonergic drugs can be detected as anxiolytics employing the Light-dark methodology (Hascoët et al., 2001).

Treatment doses	HD	R
G. glauca 125	29.3 ± 6.6*	14.5 ± 2.5*
G. glauca 250	29.5 ± 3.6*	13.3 ± 1.8*
G. glauca 500	34.4 ± 7.1*	15.1 ± 2.9*
G. glauca 1,000	35.1 ± 7.9*	12.8 ± 3.7*
G. glauca 2,000	39.1 ± 6.3*	10.4 ± 4.8*
DZP 1.0	51.0 ± 12.0*	13.4 ± 2.1*
VEH (10 µl/10 g)	13.7 ± 3.3	23.6 ± 2.8

Data presented as means ± Standard error of mean [SEM] with n = 7, *p <0.05 compared with control using Analysis of variance (ANOVA) and post-hoc Dunnett test. HD = Head-dips; R = Rearings.

Table 1. Ethological parameters recorded in the Elevated plus maze (EPM) with different doses (mg/kg) of the methanolic extract from *Galphimia glauca*

In this model, the different dosages of the standardized extract of *G. glauca* induced a significant increase in the time that the mice spent in the illuminated compartment, thus indicative of an anxiolytic effect; the data obtained with this treatment were similar to those yielded by the group of mice who received diazepam at 1.0 mg/kg (Figure 4).
With the models of the EPM and the Light/dark compartment, it was able to be demonstrated that the standardized *G. glauca* extract possesses an anxiolytic effect in the pre-clinical experimentation phase.

5.2 Anxiolytic effect produced by the isolated Galphimines

From the active extract of *Galphimia glauca*, we obtained samples of pure Galphimines. The authenticity of the G-B, G-A, and G-E compounds were confirmed by NMR spectroscopy tests of ^{1}H and ^{13}C. We also included, for comparative purposes, a sample that contained the three Galphimines together: G-B, G-A, and G-E, but without other compounds of the whole

extract (fraction of Galphimines, GRF). Intraperitoneal (*i.p.*) administration of G-B, G-A, and the GRF at a dose of 15 mg/kg produced, in male mice, a significant increase in the percentage of time that the animals spent on the open arms and percentage of number of crossings in these arms; this behavior was similar to that observed in the group that received diazepam at 1.0 mg/kg, but significantly different from that of the group that received the vehicle (10 µl/10 g of weight of Tween 20 solution at 5%) (p <0.05) (Figure 5) (Herrera-Ruiz et al., 2006B).

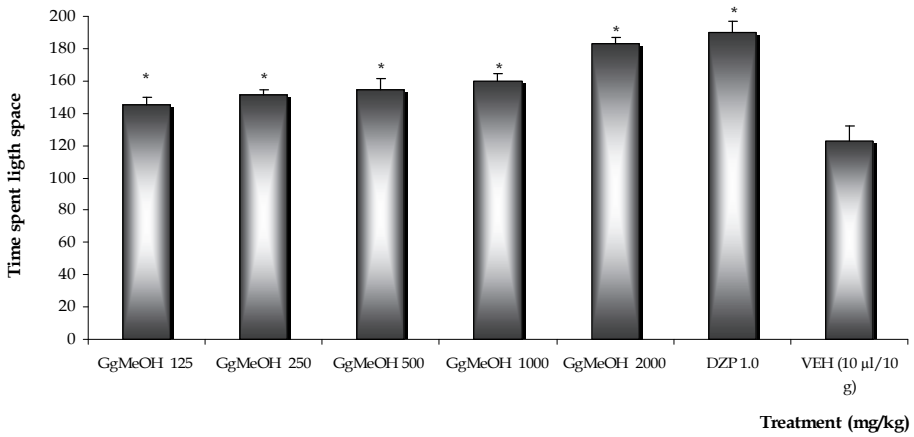

Fig. 4. Effect produced by different doses of the methanolic extract from *Galphimia glauca* on the time spent by ICR mice in the illuminated compartment in the Light-dark test. *p <0.05 with Analysis of variance (ANOVA) followed by post-hoc Dunnett test (mean ± Standard deviation [SD]; n = 7). DZP = diazepam; VEH = Vehicle (solution of Tween 20 at 5%).

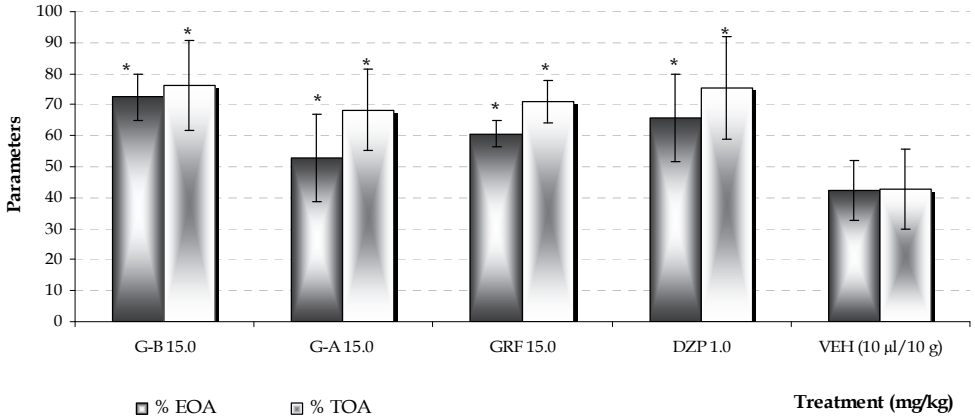

Fig. 5. Effect produced by G-B, G-A, and the pool of Galphimines on ICR mice exposed to the elevated plus-maze paradigm. *P <0.05 with Analysis of variance (ANOVA) followed by post-hoc Dunnett test (mean ± Standard deviation [SD]; n = 7). DZP = diazepam; VEH = Vehicle (solution of Tween 20 at 5%).

The ethological parameters associated with the evaluation of the number of crossings and the percentage of time on open arms was also registered for this assay. As can be observed in Table 2, the number of HD is significantly greater for animals treated with G-B, G-A, the GRF, and diazepam when statistically compared with the control (p <0.05). In agreement with these data, we observed a statistically different diminution in the R parameter for all anxiolytic treatments in comparison with the vehicle (p <0.05) (Table 2).

TX/doses	Head dips	Rearings
G-B 15	45.5 ± 12.32*	6.3 ± 2.34*
G-A 15	33.2 ± 7.31*	13.3 ± 2.94*
Pool 15	37.2 ± 7.56*	9.2 ± 3.8*
DZP 1.0	44.1 ± 10.49*	9.62 ± 4.1*
VEH (10 µl/10 g)	25.6 ± 4.53	22.2 ± 6.9

Data presented as means ± Standard error of mean [SEM] with n = 7; *P <0.05 compared with control using Analysis of variance (ANOVA) and post-hoc Dunnett test.

Table 2. Ethological parameters recorded in the Elevated plus maze (EPM) with different Galphimines (mg/kg) from *Galphimia glauca*

The data accumulated on the anxiolytic activity of the standardized extract of *G. glauca* and its triterpenic derivatives show that this Mexican medicinal species possesses effects of central depression, whose action on animal models induces behavior similar to that of substances that are clinically utilized as main therapeutic resources for treating illnesses such as anxiety, as is the case of benzodiazepines.

5.3 Action mechanism of G-B

Pure G-B has been evaluated in different pharmacological tests. These results showed that this compound, administered *i.p.* in mice, did not exhibit any significant effect as an anticonvulsant; however, it was able to increase significantly the hypnotic effect induced by sodium pentobarbital on mice in a dose-dependent manner. This compound also produced strong inhibition of the electrically-induced contraction of Guinea-pig ileum (Tortoriello & Ortega, 1993). These results suggested that the effect produced by G-B is not manifested in generalized motor processes, for example, in protection against induction of convulsion in mice. However, the pharmacological effect is observed in motor activities directed toward an objective as a goal, such as occurs with stereotypic-activity localization and adaptation in novel environments or in specific behaviors such as hypnosis induction in mice. These data supported, from that time, the idea of a selective action mechanism, particularly on regions that regulate motivational behaviors that are implicated in the processes of punishment and reward with a strong motor component. With these bases, the interaction of G-B with some of the cerebral stem structures was explored. Receptor binding experiments did not demonstrate any affinity of G-B with clonazepam, diazepam, or opioid receptors. Unexpectedly, extracellular unitary neuronal records in whole animals showed that G-B modified, with specificity, the electrical activity of ventral tegmental area (VTA) neurons in rats (Tortoriello et al., 1998). It was also observed that the inhibitory effect produced by G-B on the frequency of discharge takes place only in neurons with specific discharge patterns. The effect was observed on neurons with low frequency with periodic and rhythmic burst discharges, compatible with dopaminergic neurons. In general, G-B inhibits the activity of

the mesencephalic dopaminergic pathways. Intracellular records in brain slices showed that G-B inhibits the excitatory postsynaptic potentials; this effect was similar to that produced by GABA and clonazepam (Prieto Gómez, et al., 2003). Nonetheless, the effect produced by G-B was not blocked by bicuculline, picrotoxin, or flumazenil, thus providing an action mechanism independent of the GABA-A receptor.

6. Evaluation of the toxic activity of extracts obtained from *Galphimia glauca*

The safety of the *G. glauca* anxiolytic extract has been evaluated in pre-clinical toxicology assays using rodents under a chronic administration scheme. Determination of the possible toxic effects of this medicinal species was conducted with different extracts with the purpose of conciliating, on the one hand, the greatest efficacy of the extract with the least number of side effects, or, in the best of cases, the absence of the latter (Aguilar-Santamaría et al., 2007).

After oral administration of the 2.5 g/kg dose of the aqueous, methanolic, and ethanolic extracts of *G. glauca* in a 28-day daily scheme to Balb-C mice of both genders, it was performed an evaluation on the liver, due to that administration via oral route ensures the passage of this phytopharmaceuticals through this organ by means of the portal circulation. Based on the anatomic position between the gastrointestinal tract and systemic circulation, the liver plays an important role in the metabolism of exogenous substances (Grosse-Siestrup et al., 2002). The hepatotoxic effects produce tissue alterations accompanied by the liberation of the cellular contents into the plasma, such as the Alanine aminotransferase enzyme (ALT), the Aspartate aminotransferase enzime (AST), and the Alkaline phosphatase enzyme (ALP), which takes place after an acute inflammatory process and which is eventually related with the oxide-reduction equilibrium of the hepatocytes (Stirnimann et al., 2010). The results of the serum analysis of these enzymes, in the different groups of mice with the extracts administered, show that there are no changes in the activity of these with respect to the control group, which suggests a lack of liver damage (Figure 6).

As a complementary analysis, it is important to evaluate the neurotoxicological component by means of the observation of the Animal´s behavior. For this, it was daily observed the behavior and the physiological state of the mice, initially described by Samuel Irwin (Irwin, 1968). With this method, it was evaluated consciousness, mood state, motor activity, CNS excitation, posture, motor coordination, muscular tone, reflexes, and autonomic competence. Chronic administration of the extracts of *G. glauca* caused changes in the behavior of the mice. In special fashion, the methanolic extract produced piloerection, loss of equilibrium, and diminution in the straightening reflex, without modification of other signs, such as tearing, modification of the diameter of the pupil, respiratory movements, paralysis, and diminution of prehensile activity. Dehydration was also observed, as a consequence of treatment, which can be associated with a possible non-quantified diuretic effect, while it cannot be associated with diarrheic evacuations. These changes can be due to a potent depressor effect of the CNS produced by the prolonged administration time of the extracts at doses as high as 2.5 g/kg (Aguilar-Santamaría et al., 2007).

The therapeutic safety of the extracts of *Galphimia glauca* was also analyzed through the cytotoxicity technique on cell lines cultured *in vitro* as follows: colon cancer (HCT-15); uterine cervix cancer (UISO); human nasopharyngeal cancer (KB), and ovarian cancer (OVACAR-5). Determination of the cytotoxic effect was based on quantification of the concentration of proteins at the end of treatment (Oyama & Tagle, 1956), and results were expressed as the concentration that inhibited 50% of growth of the control treatment (EC_{50}). Values were

estimated on the semi-log graph of the concentration of the extract (µg/ml) vs. the percentage of viable cells. The data indicated that the three extracts from *Galphimia glauca* did not induce cytotoxicity on the cell lines derived from nasopharyngeal tissue (KB), uterus (UISO), and ovary (OVCAR-5), in which the EC_{50} was <2 µg/ml. The cytotoxic effect was only apparent with the colon line (HCT-15), in which ED_{50} values were 0.63, 0.50, and 1.99 µg/ml for the ethanolic, methanolic, and aqueous extracts, respectively (Aguilar-Santamaría et al., 2007).

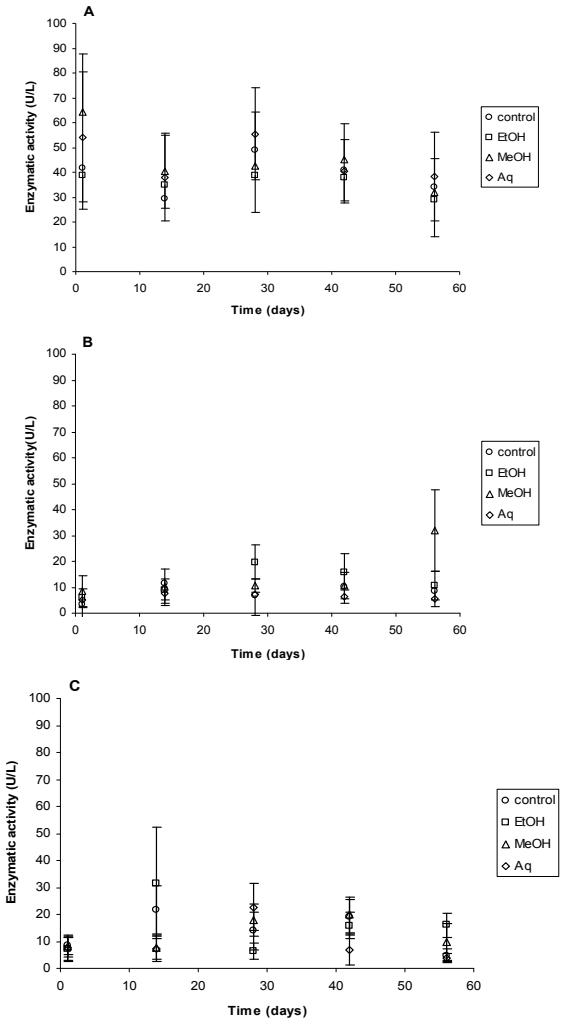

Fig. 6. Enzymatic activities in serum of Alkaline phosphatase (ALP, panel A), Alanine aminotransferase (ALT, panel B), and Aspartate aminotransferase (AST, panel C) of mice exposed for 56 days to the ethanolic, methanolic, and aqueous extracts of *Galphimia glauca*. The results are expressed as means ± Standard deviation (SD); analysis was carried out by means of Analysis of variance (ANOVA) and the Bonferroni post-test. Statistical significance was p <0.05.

Another safety parameter was evaluated by means of the genotoxicity technique in the peripheral lymphocytes of healthy volunteers; the cells were cultured with increasing concentrations of *G. glauca* extracts and the results were compared with the negative (saline solution at 0.9%) and the positive control (cyclophosphamide, 0.025 µl/ml). The culture was maintained for 72 h under standard maintenance conditions; at the end, a smear was carried out that stained with Wright stain and these were observed under the microscope (Perry & Wolf, 1974) in order to observe the sister chromatids and the presence of micronuclei; 10,000 lymphocytes were evaluated under the optical microscope with criteria defined by Fenech (2000). The data indicate that under this design, none of the extracts caused a genotoxic effect (Aguilar-Santamaría et al., 2007).
The content of Galphamines was quantified in each of the three extracts (Table 3).

Extract	Compound	Concentration (mg/g)
Aqueous	G-A	0.6
	G-B	1.034
	G-E	1.12
Methanolic	G-A	7.29
	G-B	17.47
	G-E	13.6
Ethanolic	G-A	5.35
	G-B	18.8
	G-E	17.49

Table 3. Galphmine content, extraction yield, and chromatographic analysis in High performance liquid chromatography (HPLC) of the different *Galphimia glauca* extracts

7. Effectiveness and tolerability of a phytopharmaceutical containing the standardized extract of *G. glauca* on patients with generalized anxiety disease

Based on the findings of the anxiolytic pharmacological activity of the plant species *Galphimia glauca*, and with the intention to propose a new alternative for the treatment of the generalized anxiety disorder (GAD), a phytopharmaceutical was generated from a dry extract from the aerial parts of this plant species, standardized in its G-B content (active compound). In this manner, to initiate the systematic study of the novel phytopharmaceutical, it was proposed the generation of clinical evidence on its safety and therapeutic effectiveness in patients with GAD, comparing it against a widely used drug belonging to the group of benzodiazepines (Herrera-Arellano et al., 2007).
The raw material or plant drug was obtained from a controlled culture generated by means of micropropagation in an experimental field localized in Xochitepec, Morelos, Mexico. The culture was supervised in accordance with good agricultural practices (OMS, 2003). A sample was identified by Abigail Aguilar–Contreras, M.Sc., and deposited in the Medicinal Herbarium of the IMSSM as reference. The plant raw material, leaves and stems, was dried under conditions of darkness, at room temperature, for a 2-week period; later, this was triturated and ground until we obtained 1-3-mm particles, which were stored in hermetically sealed containers until use.

The extract that was employed to formulate the phytopharmaceutical was obtained by maceration in water at 60° C for 2 h. The liquid extract was dried in two phase: the first, by distillation at reduced pressure (Heidolph, Laborota 20), and later, by a spray-dry system. The dry extract was collected and stored at 4°C until formulation of the phytopharmaceutical. Chromatographic analysis by HPLC of the extract indicated that the drying system did not modify the concentration of G-B. The final yield of the extraction process was 5%, and each g of the extract contained 1.12 mg of G-B.
The experimental treatment corresponding to the *G. glauca* pharmaceutical was formulated in hard gelatin capsules. Each capsule contained 310 mg of dry extract, at least 0.350 mg of G-B/capsule, and 500 mg of vehicle; the capsules were packed individually in blister packs of 10 units each. Several methodologies on the previously lacked drug for quality control were carried out, based on Official Mexican Norms: NOM-059-SSA1-1993 and NOM-073-SSA1-1993. Additionally, the analyses were conducted according to the Pharmacopoeia of the United Mexican States [2004]. The control treatment was formulated with 1.0 mg de lorazepam, with the same pharmaceutical presentation, packaging, and quality as the experimental drug.

7.1 Description of the clinical study

With the authorization of and registry number 2003-322-0010 of the Mexican Institute of Social Security (IMSS) Ethics and Research Committee, adult, ambulatory males and females between the ages of 18 and 65 years where included in the study. Patients must reunite the DSM IV diagnostic criteria for GAD, and a Hamilton (HAM-A) scale score ≥19 points, without pharmacological treatment in the month prior to their inclusion. In addition, the subjects could not present a history of current use, for at least 6 months previously, alcohol or drug addiction or abuse, nor data of suicidal ideation or of another psychiatric pathology that was clinically more relevant than GAD, and the signing of a letter of informed consent
Clinical, randomized, double-blind and controlled study was carried out at the Hospital General of the IMSS in Cuernavaca, Morelos, Mexico. The experimental group was treated with capsules containing the aqueous and the dry extract of *G. glauca* (standardized in 0.350 mg of G-B) at a dose of one capsule every 12 h for 4 continuous weeks. The control group received 1.0 mg of lorazepam at the same dose. The main outcome variable, therapeutic effectiveness, was considered when the HAM-A scale score was <18 points. Secondary variables included tolerability (the absence of intense or severe sedation, which merited treatment suspension) and therapeutic safety (at the end of the study, the absence of pathological alterations in the biochemical tests of hepatic and renal function: ALT; AST, serum urea, and serum creatinine). In addition, from week 1 of treatment, we implemented two scales to appraise the therapeutic effect of the treatment assigned by means of the scales denominated Global patient evaluation (GPE) and Global clinical impression (GCI). Patients were scheduled weekly on four occasions to evaluate the proposed outcomes, the presence of side effects (through a scale designed ad hoc, composed of 50 and additional items that evaluated the severity of these), treatment compliance (ingestion of at least 80% of the prescribed doses), and resupply of the assigned drug.
To evidence the differences among treatments, the Analysis of variance (ANOVA) test was utilized, a reliable statistical method for comparing continuous variables with normal distribution, while the non-parametric Wilcoxon and Mann-Whitney *U* tests were employed for comparing paired and independent data, respectively. The X^2 test served for comparing two proportions; p values ≤ 0.05 were considered as a significant difference.

7.2 Results of the clinical study in patients with GAD

The study began with 152 patients (72 in the experimental group). Table 4 compares the population characteristics on initiation of the clinical assay; significant differences were not appreciated in any of the parameters evaluated ($p \geq 0.17$). It is noteworthy that on beginning the study, the average patients age was 37.8 years, with 4.1 years of GAD disease evolution and 29 points on the HAM-A scale; in addition, feminine gender predominated with schooling equal to or greater than high school.

Variable	*G. glauca* (n = 72)		Lorazepam (n = 80)		ANOVA
	m	SD	m	SD	*P*
Age (years)	38.38	11.13	37.35	11.49	0.57
Weight (kg)	66.08	12.34	67.64	13.05	0.45
Height (cm)	158.33	9.75	157.83	7.72	0.72
BMI (kg/m^2)	26.38	4.42	27.16	4.99	0.31
SBP (mmHg)	114.58	10.99	114.59	10.050	0.96
DBP (mmHg)	72.62	9.90	72.31	9.34	0.84
CF (beats/min)	79.79	4.86	79.31	7.39	0.64
RF (resp/min)	20.98	1.975	20.96	1.99	0.94
GAD evolution (months)	51.18	56.01	48.35	59.91	0.76
	F	%	F	%	X^2 *p*
Gender					0.58
Masculine	18	25.00	17	21.25	
Feminine	54	75.00	63	78.75	
School grade reached					0.66
≤Elementary	34	47.22	35	43.75	
≥High school	38	52.78	45	56.25	
Occupation					0.88
Homemaker	26	36.11	28	35.00	
Other	46	63.89	52	65.00	
Comorbidity					0.59
Positive	24	33.33	30	37.50	
Negative	48	66.67	50	62.50	
Smoking					0.98
Positive	17	23.61	19	23.75	
Negative	55	76.39	61	76.25	
Previous alcoholism					0.17
Positive	16	22.22	11	13.75	
Negative	56	77.78	69	86.25	
Previous drug addiction					0.89
Positive	5	6.94	6	7.50	
Negative	67	93.06	74	92.50	

ANOVA = Analysis of variance; BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; CF = Cardiac frequency; RF = Respiratory frequency; GAD = Generalized anxiety disease.

Table 4. Comparison of the population characteristics on study initiation. Values correspond to means (m) and Standard deviation (SD), absolute frequencies (f), and relative frequencies (%)

During the development of the clinical assay, 38 subjects were excluded, the majority belonging to the lorazepam-treated group (21, 55.26%). Incapacitating sedation was the main reason for exclusion; this presented in 20 patients, of among whom four belonged to the group treated with the *G. glauca* phytopharmaceutical. The remaining 18 subjects were excluded due to non-drug-related reasons

For the therapeutic effectiveness analysis, 114 patients, who concluded the entire study, were included. For analyzing tolerability, 20 subjects who were excluded due to incapacitating morning sedation were added. Finally, in the therapeutic safety analysis, 113 patients who concluded the study were included, with the exception of one patient who did not allow a final blood sample to be performed for the programmed biochemical studies.

the therapeutic tolerability analysis, evaluated as morning sedation, exhibited significant differences in favor of the phytopharmaceutical (6.78 vs. 21.33%; X^2, $p = 0.01$), while none of the patients on whom we practiced the programmed laboratory tests showed pathological results; therapeutic safety was 100% in both groups.

Therapeutic effectiveness was evaluated mainly by means of the HAM-A scale, which was complemented with the EGP and the GCI. It is noteworthy that at the end of the study, both treatments significantly reduced these three scales (Wilcoxon, $p \leq 0.0001$). In Figure 7, it may be observed that the two treatments diminished with regard to the HAM-A scale from week 1 and during the subsequent 3 weeks in similar fashion (Mann-Whitney, $p > 0.54$). Likewise, on concluding the study, there were no significant differences among treatments with this scale. It is noteworthy that the phytopharmaceutical diminished HAM-A from 29 to 9 points (65.62%). In addition, at the end of the study, the phytopharmaceutical from *G. glauca* obtained 80% anxiolytic effectiveness, while lorazepam obtained 81.3%.

Figures 8 and 9 compare the effect produced by both treatments in terms of the GCI and EGP scales. It is possible to appreciate that in this analysis, the phytopharmaceutical achieved a better score with both scales, principally in weeks 2 and 3 of administration. However, the differences were not significant (Mann-Whitney *U*, $p \geq 0.09$), It is noteworthy that the phytopharmaceutical diminished the IGC from 8 to 4 points and the EGP, from 9 to 4 points.

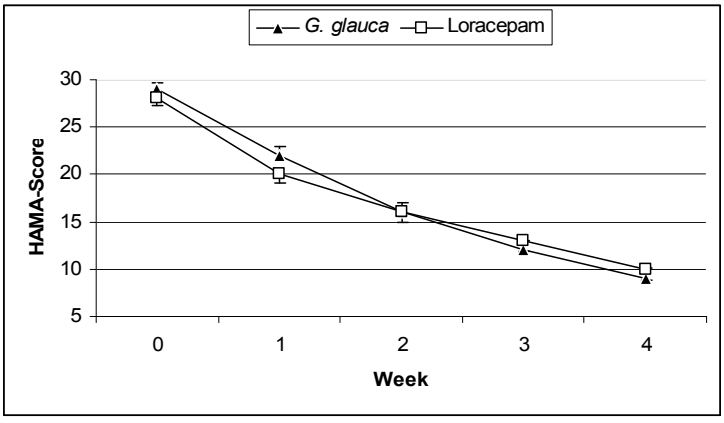

Fig. 7. The figure shows the effect of the treatments administered during 4 weeks on the Hamilton anxiety scale. Values correspond to means and Standard Error (SE) (Mann-Whitney *U*, $p \geq 0.54$).

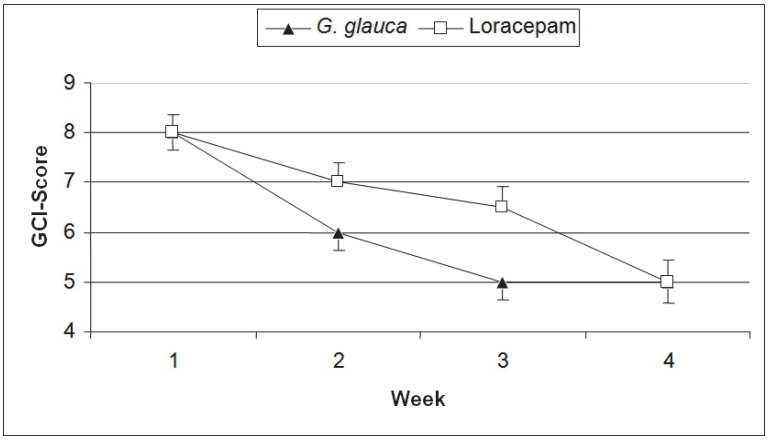

Fig. 8. Figure that shows the effect of treatments administered during 4 weeks on the global clinical impression (GCI) scale. Values correspond to means and Standard error (SE) (Mann-Whitney *U*, $p \geq 0.09$)

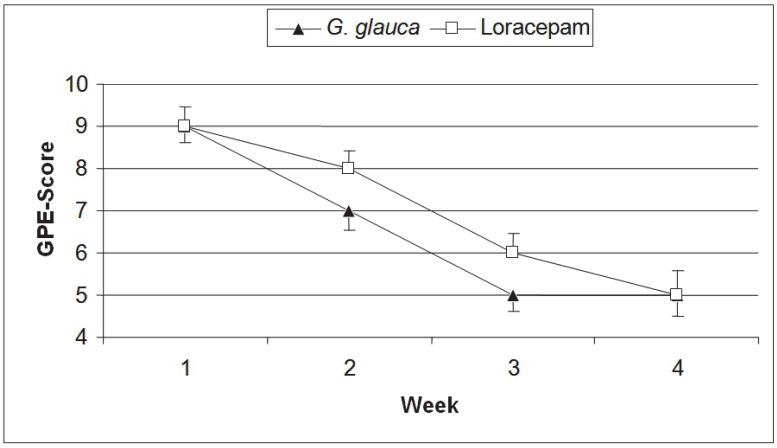

Fig. 9. Figure than shows the effect of the treatments administered during 4 weeks on the global patient evaluation (GPE) scale. Values correspond to means and Standard error (SE) (Mann-Whitney *U*, $p \geq 0.37$).

The stratified analysis, useful to appraise the effect of some confounders on the therapeutic effectiveness of the phytopharmaceutical of *G. glauca*, demonstrated that the gender of the subject, the subject's weight, and the disease evolution time did not fall into the anxiolytic effect ($p \geq 0.55$); however, age and the basal score on the HAM-A scale did exert an influence on the outcome, because subjects >38 years or with HAM-A ≤30 points showed greater percentages of therapeutic effectiveness ($p \leq 0.03$).

8. Conclusion

An innovative anxiolytic phytopharmaceutical has been developed by means of an interdisciplinary work. This product exhibited an innovative mechanism of action through an interaction with the dopaminergic system in CNS. This product was evaluated clinically by means of a double-blind clinical trial in order to compare it with lorazepam (1 mg, twice daily) in terms of therapeutic effectiveness, safety, and tolerability in patients with Generalized anxiety disorder (GAD). After 4 weeks of treatment, the phytopharmaceutical showed important anxiolytic effectiveness, very similar to that produced with lorazepam. However, regarding side effects, the phytopharmaceutical evidenced considerably higher tolerability than lorazepam.

9. Acknowledgments

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11

Psychophysiological Markers of Anxiety Disorders and Anxiety Symptoms

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1. Introduction

In anxiety research, relative few psychophysiological studies have been conducted. In this chapter, we presented previous studies that used different psychophsiological markers that can be further utilized in future research. However, there are a few things to be considered when psychophysiological markers are used in anxiety studies, first of which may be genetic factors. Genetic factors influence vulnerability to anxiety disorders. There are several genetic polymorphisms associated with anxiety disorders among which are the serotonin-transporter-linked polymorphic region (*5-HTTLPR*), the Catechol-O-methyltransferase (*COMT*), and the brain-derive neurotrophic factor (*BDNF*) gene variants. We first presented studies that invsestigated the relationship between these genetics variants and anxiety disorders. Also, it has been suggested that anxiety disorders are characterized by abnormal neural activity—amygdala hyperactivity and dysfunctional prefrontal activity—and cognitive bias favoring threat-relevant stimuli (Cisler et al., 2010; McClure et al., 2007; Nitschke et al., 2009; Whalen et al., 2008). We will present different psychophysiological markers that have been used to study dysfunctional neural, serotonergic, cognitive and autonomic activites associated with anxiety disorders. They include: (1) a loudness dependence of the auditory evoked potential (LDAEP) which is proposed to be associated with serotonin activity, (2) various components of the event-related potentials [P1, P2, N300, P3b, early posterior negativity (EPN), late positive potential (LPP), and error-related negativity (ERN)] that reflect altered neural activity in anxiety disorders and (3) the reduced heart rate variability (HRV) which indicates autonomic dysregulation associated with increased sympathetic and decreased vagal control of the heart. Particularly, in this chapter, we introduced the loudness of the auditory evoked potential (LDAEP) as a possible psychophysiological marker that can be utilized in anxiety research. Our previous studies revealed that patients with different subtypes of anxiety disorders produced distinctive LDAEPs and that the LDAEP could play an important role in predicting the efficacy of selective serotonin reuptake inhibitor (SSRI) treatment in anxiety disorders (Park et al., 2010, 2011). We suggest that utilizing the LDAEP along with other various ERP components indicating neural and cognitive dysfunctions associated with anxiety disorders may enhance our understanding of the etiology and maintenance of anxiety disorders. Also, it is important to understand how they interact with each other and with other environmental stressor to reinforce or to exacerbate anxiety symptoms (see Figure 1). Of clinical relevance is whether these psychophysiological markers may play a role in predicting clinical outcome of different treatment.

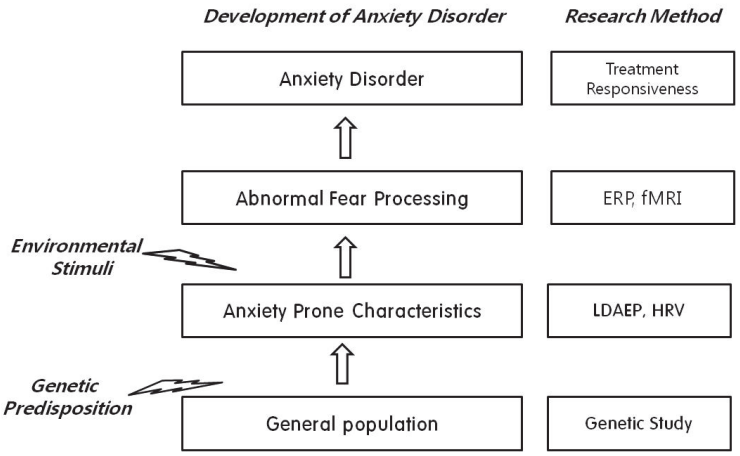

Fig. 1. Development of anxiety disorders and research methods that can be used in each stage.

2. Genetic predispositions of anxiety symptoms

Anxiety disorders have genetic predispositions and it is critical to consider individuals' genetic predispositions to develop anxiety prone characteristics and anxiety disorders. Several anxiety-related genetic markers have been identified, one of which includes the serotonin transport promoter polymorphism (*5-HTTLPR*). The dysfunctional serotonergic system (5-hydroxytryptamine or *5-HT*) is known to be implicated in anxiety and fear (Harmer et al., 2004). In human, serotonergic raphe neurons project to different brain structures (e.g., cortex, amygdala, hippocampus) and are associated with integrating various functions including emotion, cognition, motor function, pain, circadian and neuroendocrine functions such as food intake, sleep and sexual activity (Lesch et al., 1997). The 5-HT transporter (*5-HTT*) plays a vital role in regulating serotonergic neurotransmission by facilitating the reuptake of 5-HT from the synaptic cleft (Lesch et al., 1996; Hariri et al., 2002). Lesch and colleagues (1996, 1997) identified a relatively common polymorphism in the promoter region of the serotonin transporter gene, which results in two different alleles—the short (*s*) and long (*l*). Research has showed that the *5-HTTLPR* plays a functional role in regulating *5-HTT* expression and *5-HTTLPR* genotype may modulate *5-HTT* expression (Lesch et al., 1996; Lesch et al., 1997). Individuals carrying one or two copies of the *s* form of *5-HTTLPR* were associate with almost 50% reduction in *5-HTT* availability compared to individuals homozygous for the *l* variant (Lesch et al., 1996; Lesch et al., 1997; Hariri et al., 2002). As a result, it has been reported that *s*-carriers were associate with increased anxiety-related behaviors and greater risk for developing anxiety in stressful life situations compared to individuals homozygous for the *l* variant (Lesch et al., 1996; Lesch et al., 1997; Hariri et al., 2002). Research also indicated that allelic differences in the 5-HTT may modulate activity of neural circuits (Heinz et al., 2005; Pezawas et al., 2005). Health individuals carrying one or two copies of the *s* allele showed greater activity in the amygdala in response to fearful stimuli (Heinz et al., 2005). Also, individuals with the *s* allele showed altered coupling of prefrontal-amygdala feedback circuit—which may lead to dysfunctional amygdala regulation in response to fearful stimuli—compared to individuals homozygous of the *l* allele (Heinz et al., 2005; Pezawas et al., 2005).

Another gene associated with anxiety disorders is the Catechol-O-methyltransferase (*COMT*) genetic variation (Funke et al., 2005). *COMT* is an enzyme that plays an important role in the metabolism of brain dopamine and norepinephrine (Gadow et al., 2009). The *COMT* gene can be found in chromosome 22q11 and contains several single nucleotide polymorphisms (SNPs) that are functionally important. For example, *Val158Met* (rs4680)—associated with encoding either valine (*Val*) or methionine (*Met*)—plays an important role in modulating *COMT* activity in the prefrontal cortex (Harrison et al., 2008). Current evidences suggest that *Val158Met* may be associated with anxiety disorders, particularly bipolar disorder, via controlling dopamine activity in the prefrontal cortex (Funke et al.,2005). Individuals with Val158 homozygous showed 35-50% higher *COMT* activity in human dorsolateral prefrontal cortex than those with Mel158 homozygous (Harrison et al., 2008). Although *Met-COMT* is considered to play an important role in the development of bipolar disorder, there exists evidence that *Val-COMT* is also associated with bipolar disorder (Funke et al., 2005).

Lastly, the brain-derived neurotrophic factor (*BDNF*) gene variants are suggested to be linked with anxiety disorders (Chen et al., 2006; Gadow et al., 2009). *BDNF* is a neurotrophin that plays an important role in neuronal growth, differentiation, and synaptic plasticity (Chen et al., 2006; Gadow et al., 2009; Rasmusson et al., 2002). BDNF is also associated with learning and memory and modulates aggression (Rasmusson et al., 2002). It has been reported that BDNF plays a role in mediating effects of stress (Rasmusson et al., 2002). Reduced BDNF expression in the hippocampus was observed in response to stress, which may contribute to hippocampus-dependent memory deficits and the decreases in hippocampal volume associated with patients with post-traumatic stress disorder (PTSD; Rasmusson et al., 2002). Recent studies investigated the relationship between a SNP in the *BDNF* gene, *Val66Met*, and psychopathology, which yielded conflicting results (Jiang et al., 2005). In an animal study, when exposed to stress, *BDNF* Met/Met mice demonstrated anxiety-related behaviors and were not responsive to the antidepressant, fluoxetine (Chen et al., 2006). Studies showed that *Val66* allele were associated with greater neuroticisim scores, suggesting that individuals with the Val allele may have increased risk for developing anxiety or depression (Hünnerkopf et al., 2007; Sen et al., 2003). However, no association between *BDNF Val66Met* genotypes and neuroticisim was observed in Asian female participants (Tsai et al., 2004). *BDNF Met66* allele was found to be a risk allele for anxiety and depression (Jiang et al., 2005) whereas other found it to be a protective allele for obsessive-compulsive disorder (OCD; Hall et al., 2003). In sum, *BDNF* may be related to anxiety disorder though it is yet to be determined which specific variant is responsible for the pathogenesis of anxiety disorders (Gadow et al., 2009).

So far, we have presented different candidate genetic marker of anxiety disorders. To have better understanding of anxiety disorders, it would be important to identify genetic polymorphisms associated with anxiety disorders and study together with psychophysiological markers which will be discussed later.

3. Neurophysiological and cognitive characteristics of anxiety disorders

Anxiety disorders are characterized by altered neurophysiological and cognitive functions. Various psychophysiological markers used in anxiety research may reflect these altered neural and cognitive characteristics of anxiety disorders. Here, we briefly described altered neural activity and dysfunctional cognitive processing of threat-relevant information in people with anxiety disorders.

3.1 Amygdala hyperactivity and reduced PFC function

Amygdala hyperactivity has been considered as an important neural characteristic of anxiety disorders (Bar-Haim et al., 2005; Dannlowski et al., 2007; McClure et al., 2007). Previous functional brain imaging studies indicated that anxiety disorders are linked with hyperactivity of the amygdala in response to anxiety provoking tasks (e.g., public speaking), fear-conditioning, and viewing face pictures with emotionally negative expressions (Phan et al., 2006). Also, functional magnetic resonance imaging (fMRI) studies revealed that effective treatment produced significantly reduced amygdala activity in patients with social phobia (Kilts et al., 2006; Furmark et al., 2002). Patients characterized with greater amygdala hyperactivity before treatment responded better to treatments such as SSRI medications and cognitive behavioral therapy (CBT; McClure et al., 2007). More recently, fMRI studies also revealed that high-trait anxiety individuals showed reduced activity in anterior cingulate cortex (ACC)—associated with conflict monitoring—and lateral prefrontal cortex (lateral PFC)—related with attentional control over threat-relevant distractors (Bishop et al., 2004). Patients with generalized anxiety disorder (GAD) patients who showed greater activation in ACC in response to or in anticipation of aversive pictures were associated with better treatment outcome (Nitschke et al., 2009; Whalen et al., 2008). In addition, GAD patients who showed greater activation in the ventrolateral prefrontal cortex—associated with emotional regulation by exerting inhibitory control over subcortical structures—had fewer anxiety symptoms (Monk et al., 2006). Therefore, anxiety disorders may be characterized by amygdala hyperactivity—associated with heighten sensitivity to motivation-relevant stimuli—and reduced PFC activity—resulting in the lack of top-down attentional control and emotional regulation (Bishop et al., 2004; McClure et al., 2007; Monk et al., 2006).

3.2 Cognitive characteristics of anxiety disorders

It has been well established that anxious individuals exhibit attentional biases toward threat-relevant stimuli (Cisler et al., 2010; Mathews et al., 1997). Anxiety-related attentional biases are typically observed in three different ways: (1) faster detection to threat-relevant stimuli relative to nonthreat stimuli, (2) difficulties in disengaging attention away from threat stimuli (sustained attention to threat stimuli), and (3) attentional avoidance of where threat-relevant stimuli are presented (Cisler et al., 2010; Fox et al., 2001; Koster et al., 2004; 2005). Initially, anxiety-related attentional biases were studied in the emotional Stroop task. In the task, threat or neutral words were written in different colors and participants were instructed to name the color of ink while ignoring the meaning of the word (Cisler et al., 2010). Research showed that high-trait anxiety participants were slower to name the color of ink in which threaten words were written, particularly to items relevant to their anxiety conditions. For instance, Vietnam combat veterans with Post-Traumatic Stress Disorder (PTSD) and without PTSD were asked to name the color of PTSD-related words,OCD-related words, positive words, and neutral words (McNally et al., 1993). Veterans with PTSD took longer to name the color in which PTSD-related words were written relative to veterans without PTSD who showed no difference in reaction times across different types of the words. Similarly, slower responses were observed to read threat-relevant words in patients with GAD (Mathews & MacLeod, 1985) and panic disorder (McNally et al., 1994). Highly anxious non-clinical participants showed negativity bias even though they could not consciously aware of the presence of threat-relevant stimuli (MacLeod and Rutherford, 1992). Moreover, performances on the masked emotional Stroop task predicted later emotional reactivity (Den Hout et al., 1995).

Fox and her colleagues (2001) adapted Posner's spatial cuing task to systematically investigate different components of anxiety-related attention bias (Posner & Petersen, 1990). In the spatial cuing task, a target is preceded by a cue which can be either "central" (e.g., an arrow presented at the center of the display pointing one of two peripheral boxes in which the target would subsequently appear), or "peripheral" (e.g., an abrupt luminance of one of the peripheral boxes; Posner et al., 1990; Posner et al., 2007; Bartolomeo et al., 2001). When the cue appears on the same display that a target appears, it is considered to be a valid trial because the cue correctly predicts the location in which the target appears. However, when the cue fails to predict where the target will appear, it is considered to be invalid. In valid trials the detection of targets is facilitated (cuing benefits) whereas the detection of targets is delayed in invalid trials (cuing costs). In the modified emotion spatial cuing task, emotionally charged words or pictures were used as cues (Fox et al., 2001; Vuilleumier et al., 2009). If high-trait anxiety participants automatically draw their attention to threat-relevant stimuli, their response to targets following valid threat-relevant cues should be fast (Fox et al., 2001). If high-trait anxiety participants have a difficulty in disengaging their attention from threat-relevant stimuli, then their responses to targets following invalid threat-related stimuli should be slow (Fox et al., 2001). In the first study, Fox et al. (2001) found that attentional disengagement from threat-relevant words took longer compared to neutral or positive words. However, there was no difference between high and low-trait anxiety participants. In the second study, they used schematic faces with 'angry,' 'neutral,' and 'happy' facial expressions. When a cue duration period increased to 250 ms, only high anxious individuals showed the delayed attentional disengagement from angry faces. Fox and colleague (2001) suggested that regardless of anxiety level, people were initially drawn to threat-relevant information for a brief period of time (about 100 ms). However, low-trait anxiety participants were capable of quickly disengaging their attention from threat-relevant, positive and neutral information whereas high-trait anxiety participants were less successful in disengaging their attention from the location in which threat-related information was presented.

Recent studies suggested that high-trait anxiety is associated with the "vigilance-avoidance" attentional patterns in response to threat-relevant information, which may account for the maintenance of anxiety (Koster et al., 2006). The initial attention to mildly and highly threatening information may trigger the constant processing of fearful information and interfere with engaging in goal-directed behaviors (Koster et al., 2006). Faster detection of mildly and highly threatening information trigger anxious conditions in high-trait anxiety participants, which reinforces them to avoid threatening information in an attempt to reduce anxiety (Koster et al., 2006). However, this strategic attentional avoidance may not be a good coping strategy because it can lead to a failure of habituation to threaten stimuli and constantly remind of fear (Koster et al., 2006). Koster and colleagues (2005, 2006) used neutral, mildly and highly threatening pictures as cues and reported that when picture cues were presented at 100 ms, high-trait anxiety participants exhibited faster attentional engagement and slower attentional disengagement in response to highly threatening pictures compared to low-trait anxiety participants. However, when picture cues were presented longer, 500 ms, high-trait anxiety participants showed slower attentional engagement to highly and mildly threatening cues, which may suggest attentional avoidance to highly threatening stimuli (Koster et al., 2006).

Interestingly, there is evidence suggesting that the *5-HTTLPR s* allele—genetic predispositions to anxiety—may be related with anxiety-related cognitive bias (Beck, 2008). Twenty-seven psychiatric inpatients who carried *s* allele of the promoter region of the *5-HTTPR* showed anxiety-related attentional biases favoring threat-relevant words compared to patients with homozygous for the *l* variant (Beevers et al., 2007). Fox and colleagues recently showed that healthy individuals with homozygous for the *l* allele were characterized by a marked avoidance of negative stimuli and a vigilance for positive stimuli whereas *s*-allele carriers did not show such protective attentional pattern (Fox et al., 2009). Therefore, allelic variation of the promoter region of the serotonin transporter gene may influence the way in which an individual processes emotional materials.

4. The event-related potentials (ERP) components used to study anxiety disorders

Several ERP components have been used to study serotonergic, neural, and cognitive dysfunctions associated with anxiety disorders. We provided underlying mechanisms of the loudness dependence of the auditory evoked potential (LDAEP) and presented studies that used the LDAEP in anxiety research. Also, researchers have studied other ERP components that reflect neural mechanisms of cognitive bias toward threat-relevant stimuli commonly observed in patients with anxiety disorders.

4.1 Loudness of the auditory evoked potential

It has been proposed that the LDAEP—which measures activity in the primary auditory cortex in response to different tone intensities—indicates the functioning of the central serotonergic system (Hegerl et al., 1993; Juckel et al., 1999). More specifically, the LDAEP is defined as the linear regression slope calculated from five amplitudes of N1/P2 components in response to increasing five auditory tones (Senkowski et al., 2003; see Figure 2). Research has indicated that the LDAEP is inversely related to central serotonergic activity: a stronger LDAEP indicates lower serotonergic neurotransmission and vice versa (Juckel et al., 1999, Park et al. 2010).

Initial evidence that linked the LDAEP and the serotonergic system came from animal studies (O'Neill et al., 2008). Administrating quipazine maleate—a 5-HT_2 receptor agonist—reduced the amplitude of N1/P2 components whereas administrating spiperone—a 5-HT_{1A} receptor antagonist—increased the N1/P2 amplitude in rats (Manjarrez et al., 2005). Administrating the precursor of 5-HT, L-tryptophan, was associated with the reduced amplitude of N1/P2 components (Manjarrez et al., 2005). Other studies showed that the LDAEP was inversely correlated with the concentration of 5-hydroxyindoleacetic acid (main metabolite of serotonin) in cerebrospinal fluid (von Knorring et al, 1981). High scores in the serotonin syndrome scale were associated with weaker LDAEPs and vice versa in depressive patients who underwent SSRI treatment (Hegerl et al., 1998). Individuals scored high on measures of sensation seeking and impulsiveness were associated with stronger LDAEP—potentially indicating reduced serotonergic function (Brocke et al., 2000; Hegeral et al., 1995). So far, there have been three studies that investigated the relationship between allelic variants of the seroton transporter gene and the LDAEP. It has been found that individuals homozygous for the *l* variant exhibited lower LDAEP (Gallinat et al., 2003) whereas others (Strobel et al., 2003; Hensch et al., 2006) reported that the *l* allele carriers

showed stronger LDAEP. These studies provide evidence that the LDAEP is linked with the serotonin transporter polymorphism although there are inconsistencies in predicting directional changes in serotonin neurotransmission (O'Neill et al., 2008).

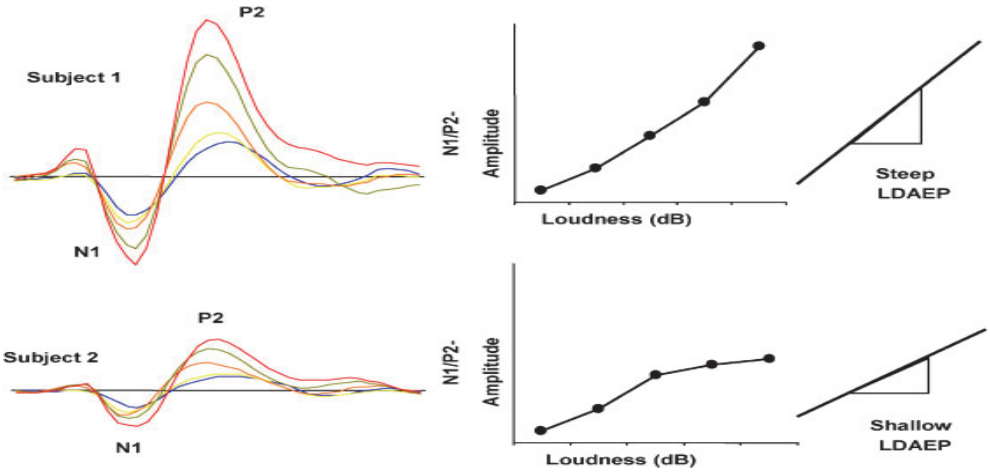

Fig. 2. Subject 1 possesses a steep LDAEP (a large increase in N1/P2 amplitude with increasing loudness) whereas subject 2 shows a shallow LDAEP (a small increase in N1/P2 amplitude with increasing loudness). Adapted from O'Neill et al., 2008. (Reprinted by permission of author and publisher).

There is evidence suggesting that the LDAEP is also modulated by dopaminergic neurotransmission (Juckel et al., 2008). High intensity dependence of auditory and visual evoked potentials were associated with low levels of dopamine metabolites (i.e., homvanillic acid) in cerebrospinal fluid and urine (Pogarell et al., 2004; O'Neill et al., 2008). Pogarell and colleagues (2004) used single photon emission computed tomography (SPECT) and showed that the LDAEP was positively associated with both serotonin and dopamine transporter availabilities in patients with OCD. Recently, Juckel and colleagues (2008) found that the LDAEP is also related with the genetic variants of the c*OMT*—implicated in the inactivation of synaptic dopamine (Stein et al., 2005; Samochowiec et al., 2004). Reduced *COMT* activity caused by genetic polymorphisms was associated with a weaker LDAEP (Juckel et al., 2008).

The LDAEP has been utilized to study dysfunctional serotonergic and dopaminergic activity in patients with GAD (Senkowski et al., 2003), PTSD (Park et al., 2010), schizophrenia (Juckel et al., 2003) or depression (Gallinat et al., 2000). Recently, Park and colleagues (2010) compared the results of the LDAEP in a variety of psychiatric patients including GAD, PTSD, panic disorder, bipolar depression, major depressive disorder (MDD), and schizophrenia. Individuals with different anxiety disorders produced different strengths of LDAEPs (see Fig. 3), which raised a possibility that the differences in the LDAEP may be associated with distinctive anxiety symptoms and cognitive impairments that characterize different subtypes of anxiety disorders. However, further studies are needed to explicate the relationship between different anxiety disorders and the strength of LDAEP.

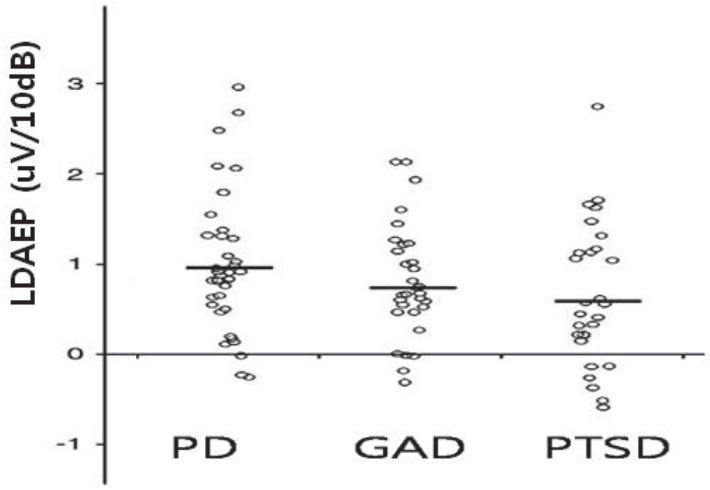

Fig. 3. Comparison of the LDAEP among panic disorder (PD), generalized anxiety disorder (GAD), and post-traumatic stress disorder (PTSD). Note: Adapted and modified from Park et al. (2011). (Reprinted by permission of publisher).

Furthermore, evidence suggests that the LDAEP can serve as a predictor of responses to SSRI treatment in GAD patients, which phenomenon was previously observed in patients with MDD (Gallinat et al., 2000; Linka et al., 2004). Research has indicated that a strong LDAEP— indicating lower serotonergic activity and turnover rate—is associated with a favorable response to SSRI treatment in patients with depression (Gallinat et al., 2000; Linka et al., 2004). Our study (Park et al., 2011) also showed that GAD patients who had stronger LDAEPs responded favorably to SSRI (escitalopram) treatment. Park et al. (2011) also confirmed this finding in the brain source activity of the LDAEP, which was measured using a standardized low resolution brain electro-magnetic tomography (*sLORETA*; Pascual-Marqui, 2002). GAD patients who showed greater loudness dependence source activity in the primary auditory cortex were more responsive to the escitalopram treatment (see Fig. 4). The study (Park et al., 2011) implies that source activity of the LDAEP, as well as the cortical LDAEP, may play an important role in predicting the efficacy of SSRI treatment in GAD patients, which can be used in clinical settings.

4.2 Other ERP components that are associated with neural mechanisms of cognitive bias

Because of high-temporal resolution, the event-related potentials (ERP) method can be particularly useful to capture the time course of anxiety-related attentional biases (Mercade et al., 2009) and has been utilized in some studies (Holmes, et al., 2008; Bar-Haim et al., 2005). Researchers have studied the P1—the first major positive voltage deflection occurring 50-165 ms after the onset of stimulus—and the early posterior negativities (EPNs)—showing negative deflection over the temporo-occipital sites within a time window between 150 (200) and 300 ms—in response to emotional stimuli (Schupp et al. 2006; see Figure 5). A number of studies reliably found that negative faces elicited the significantly higher amplitude of the

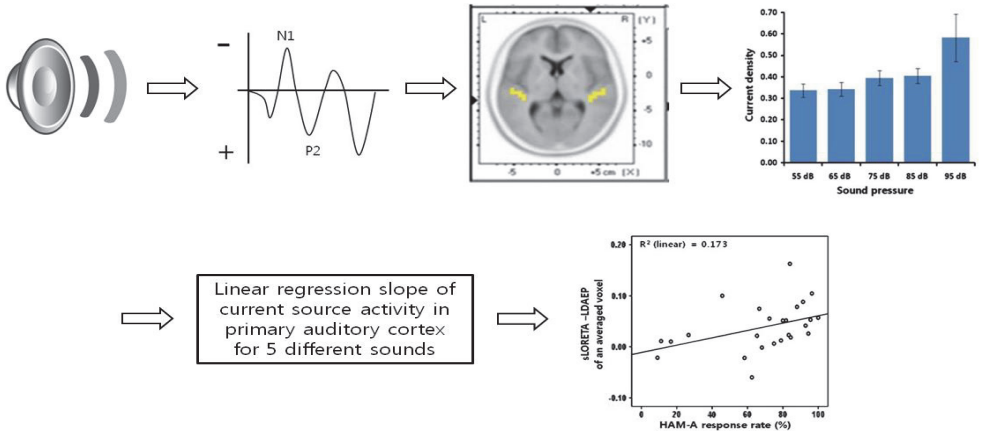

Fig. 4. The source activities of ERP components (N1-P2) on the primary auditory cortex were obtained by the *sLORETA* program. And the linear regression slope of the source activities of five ERP components was considered as the sLORETA - loudness dependence of the auditory evoked potential (*sLORETA-LDAEP*). The *sLORETA-LDAEP* showed the significant positive correlation [Spearman's rho = 0.54 (p=0.005)] with symptom response rates measured by Hamilton Anxiety Rating Scale (HAM-A) in patients with generalized anxiety disorder treated with escitalopram. Note: Adapted and modified from Park et al. (2011). (Reprinted by permission of publisher).

exogenous visual P1 component (Pourtois et al., 2005; Holmes et al., 2008). Greater EPNs were observed in response to negative faces compared to positive and neutral faces over lateral posterior and occipital areas (Schupp et al., 2004b; Holmes et al., 2008). Similarly, Bublatzky and colleagues (2010) reported that emotional pictures elicited an enlarged EPN. The P1 and the EPNs are particularly useful because they are associated with the preferential attentional processing of negative facial expressions in extrastriate visual cortex, which is extensively modulated by the amygdala and attentional networks in fronto-parietal cortex (Homles et al., 2008). Also, the EPN may be associated with a transient stage at which motivationally relevant stimuli are 'tagged' for prioritized processing, which can be useful to study preferential processing of motivationally relevant stimuli commonly observed in patients with anxiety disorders (Cuthbert et al. 2000; Michalowski et al. 2009; Schupp et al., 2006). In Holmes et al. (2008), high-trait anxiety participants who performed a variant of the emotional spatial cuing task showed an enhanced early P1 component to fearful faces relative to neutral faces at occipital electrode sites (Holmes et al., 2008). However, high-trait anxiety participants did not show greater lateral parietal negativities (or EPNs) in response to fearful faces, which may indicate attentional avoidance following the initial attentional vigilance or the failure to differentiate threat from non-threat stimuli (Holmes et al., 2008). In contrast, Wieser and colleagues (2010) reported that that healthy participants who expected to make public speaking produced enhanced EPN responses for angry facial expressions, suggesting enhanced early perceptional processing of angry faces.

Furthermore, Bar-Haim and colleagues (2005) used the emotional spatial cuing task similar to Fox et al., (2001) and reported high-trait anxiety participants had greater amplitudes of

the P2 component—the following major positive voltage deflection occurring 50-165 ms after the onset of stimulus—to angry faces compared to low-trait anxiety participants. Greater P2 components indicate greater attentional allocation to threat-related stimuli, which is frequently exhibited in individuals with anxiety disorders (Holmes et al., 2008).
Rossignol and colleagues (2005) used an emotional oddball task in which participants were asked to detect an infrequent emotional target stimulus among a series of frequent neutral standard stimuli and provided evidence that anxiety modulated the amplitude of N300, a negative deflexion peaking at central sites around 300ms, and the latency of the P3b component, occurring at parietal situes around 450 ms. N300 is associated with affective processing and P3b reflects decision-making and premotor response-related stage (Rossignol et al., 2005). High-trait anxiety participant showed the reduced amplitude of N300 suggesting that they were less able to process the emotional content of faces. However, faster detection of infrequent emotional target stimuli as suggested by faster reaction time latency and the P3b latency indicated that high-trait anxiety participants made fast decisions and preparation for actions (Rossignol et al., 2005).

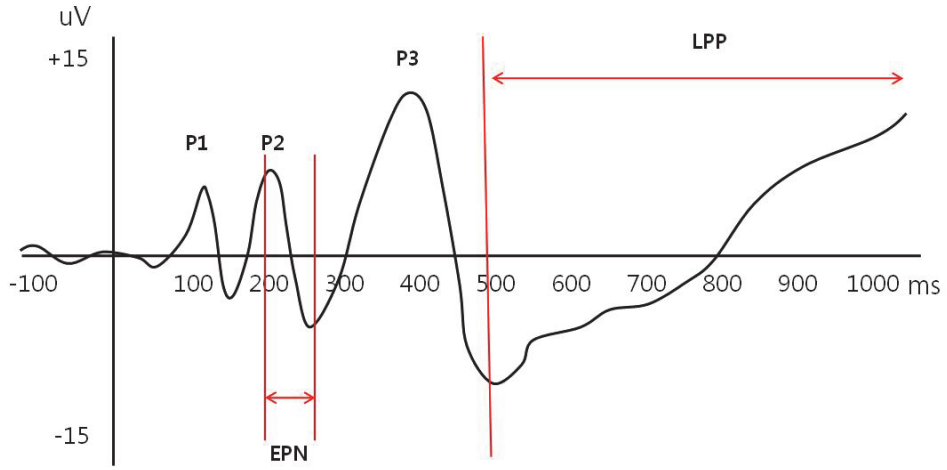

Fig. 5. Illustration of P1, P2, P3, early posterior negativity (EPN), and late positive potential (LPP) in response to fearful target stimuli in the odd ball task.

Another ERP component that has been used to study the abnormal processing of threat-relevant stimuli in anxious individuals is the late positive potential (LPP)—which becomes apparent approximately 300 ms after stimulus onset (Hajcak et al., 2010). Research indicated that greater LPPs are observed in response to emotional compared to neutral stimuli and do not habituate to stimuli that are repeatedly presented (Cuthbert, Schupp, Bradley, Birbaumer, & Lang, 2000; Dillon, Cooper, Grent-'t-Jong, Woldorff, & LaBar, 2006; Foti, & Hajcak, 2008; Hajcak, Dunning, & Foti, 2007; Hajcak & Nieuwenhuis, 2006; Hajcak & Olvet, 2008; Moser, Hajcak, Bukay, & Simons, 2006; Schupp et al., 2000; Schupp, Cuthbert et al., 2004a; Schupp, Ohman et al., 2004b; Schupp, Junghöfer, Weike, & Hamm, 2003). The LPP is associated with sustained attention toward, and elaborative processing of, motivationally relevant stimuli, which phenomenon is commonly observed in patients with anxiety

disorders (Hajcak et al., 2010). The source activity of the LPP may be traced to occipital activation resulting from elevated amygdala activity to motivationally relevant stimuli (Hajcak et al., 2010). It has been suggested that the LPP may reflect activity in the locus coeruleus (LC)-Norepinephrine (NE) system that innervates large areas of the cortex in response to motivationally relevant stimuli (Hajcak et al., 2010). Research indicated that patients with anxiety disorders had larger LPP than health controls (Leutgeb et al., 2010; MacNamara & Hajcak et al., 2010). For instance, spider phobia patients showed enhanced LPP amplitude in response to spider pictures (Leutgeb et al., 2010). Also, GAD patients had larger LPPs to aversive targets presented with neutral distrators compared to healthy controls (MacNamara & Hajcak, 2010).

There is an ERP component that can reflect prefrontal activity. For example, research has indicated that the error-related negativity (ERN), a negative deflection observed at fronto-central sites, is generated in the anterior cingulate cortex (ACC; Olvet and Hajcak, 2008). The ERN arises around the time when an erroneous response is made and peaks at 50-100 ms after stimulus onset (see Figure 6). The ERN has been found across different types of the tasks that employed various stimuli and response modalities (Hajcak et al., 2003; Weinberg et al., 2010). Significantly larger ERN component has been associated with anxiety (Xiao et al., 2011). Undergraduate students who scored high on the Penn State Worry Questionnaire

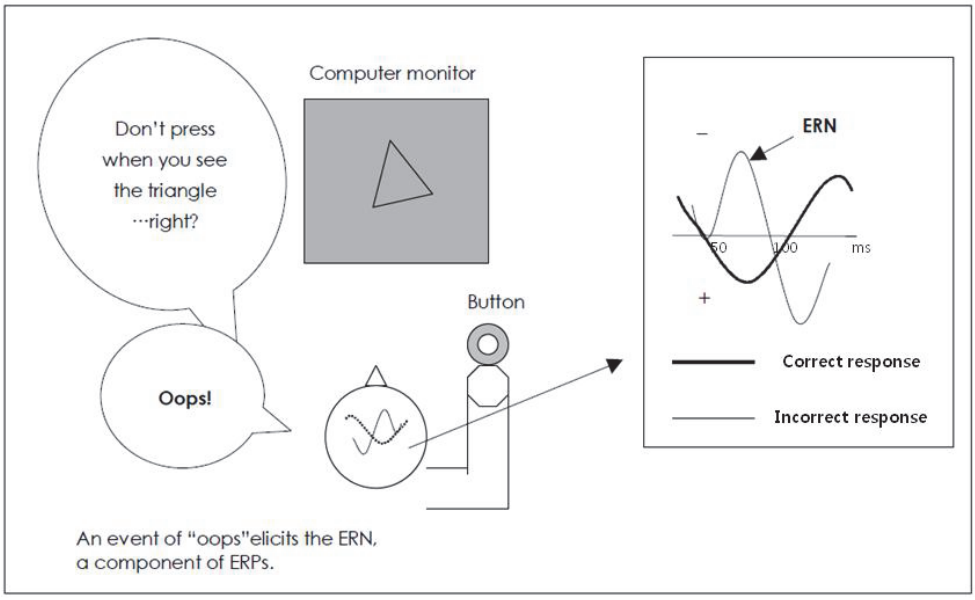

Fig. 6. Incorrect response is involved with the generation of ERN (error-related negativity). The ERN is negative-going deflection in averaged electrical brain activity that is time-locked to the execution of an incorrect response. Note that the ERN is absent for correct response. Note: Adapted and modified from Kim and Lee (2008). (Reprinted by permission of author and publisher).

Had significantly greater ERN compared to both phobic and non-anxious participants when making errors in the Stroop task (Hajcak et al., 2003). Also, significantly larger ERN

amplitudes were observed in individuals who had high scores on negative affect scores on negative affect compared to those with low scores on NA (Hajcak et al., 2004). Administrating anxiolytics such as oxazepam and alprazolam has decreased ERN amplitudes (Johannes et al., 2001; Riba et al., 2005). Several studies showed that patients with OCD have been associated with enhanced ERN amplitudes which did not change after successful treatment (Endrass et al., 2010; Gehring et al., 2000; Hajcak et al., 2008). A recent study showed that GAD patients showed larger ERN relative to healthy controls (Weinberg et al., 2010). Olvet and Hajcak (2008) have proposed that error monitoring activity of the ACC indexed by the ERN may play a role as an endophenotype of anxiety disorders.

5. Heart rate variability

Patients with anxiety disorders are characterized by reduced heart rate variability (HRV; Ost et al., 1984; Friedman, 2007). HRV—which refers to the differences in beat-to-beat alterations in heart rate—indicates the dynamic interplay between sympathetic and parasympathetic (vagal) activity in the heart (Berntson et al., 1997; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Thayer and Lane, 2000). Under resting conditions, the heart is predominately under the control of the parasympathetic activity (Levy, 1971). Although the intrinsic heart rate is approximately 105 beat per minute, resting heart rate is only 6o-80 beats per minute, indicating that the heart is under the strong vagal control ("vagal dominance"; Brownley et al., 2000; Ellis & Thayer, 2010)

There is converging evidence suggesting that reduced HRV—indicating autonomic dysregulation associated with elevated sympathetic activity and reduced vagal activity of the heart—is commonly observed in patients with panic disorder, GAD, and even children of patients with panic disorder (see Friedman, 2007 for a review; Friedman and Thayer, 1998; Srinivasan et al., 2002). Research has indicated that reduced HRV is associated with predispositions to various physical and psychological illnesses and considered to be a predictor of all-cause mortality (Thayer and Lane, 2000). Also, reduced HRV is associated with reduced attentional control, poor emotional regulation, decreased response to various stimuli, and antisocial behavior in adolescents (Mezzacappa et al., 1997; Friedman, 2007; Thayer et al., 2000). Patients who experienced severe panic attacked frequently exhibited reduced HRV in various situations (e.g., quiet rest, shock avoidance, face immersion, isoproterenol infusions; Friedman et al., 1993; Yeragani et al., 1995). High trait anxiety was associated with autonomic dysreguation indexed by reduced HRV (Miu et al., 2009). Thus, reduced HRV may play a critical role in the development of anxiety disorders and can be considered as an important endophenotype of anxiety (Friedman, 2007; Crişan et al., 2009). In a recent review of the literature, Friedman (2007) provided a number of studies that linked reduced HRV with a variety of anxiety disorders over the past 15 years and provided the summary of the Neurovisceral Integration model of anxiety.

5.1 The Neurovisceral Integration Model of anxiety

Several researchers have identified neural networks in the central nervous system associated with autonomic, emotional, and cognitive self-regulatory responses, one of which is the central autonomic network (CAN; Benarroch, 1993; Thayer and Lane, 2000; 2002). The structures of the CAN include the anterior cingulate, insula, ventromedial prefrontal cortices, the central nucleus of the amygdala, the paraventricular and related nuclei of the

hypothalamus, the periaquaductual gray matter, the parabrachial nucleus, the nucleus of the solitary tract (NTS), the nucleus ambiguous, the ventrolateral medulla, the ventromedial medulla, and the medullary tegmental field (for reviews, Ellis and Thayer, 2010; Thayer and Lane, 2002). Reciprocally interconnected components in the CAN allow information to flow in both top-down and bottom-up fashions (Theyer and Lane, 2000, 2002). Also, these components are loosely connected so that it is possible to recruit additional structures when it is necessary to make specific behavioral adaptations (Thayer and Lane, 2000, 2002).

In the CAN, the prefrontal cortical structures—including the orbitofrontal cortex (OFC) and medial prefrontal cortex (mPFC)—modulates cardiovascular, autonomic, and endocrine responses by exerting tonic inhibitory control on subcortical structures, such as the central nucleus of the amygdala (Thayer and Lane, 2000; Thayer et al., 2009). In emotionally stressful and threatening situations, sympathoexcitatory subcortical circuits are activated to produce the fight or flight response (Thayer and Lane, 2000; Thayer and Seigle, 2002). However, the constant activation of sympathoexcitatory subcortical activity is not suitable for many other situations and will eventually wear and tear the system down. Therefore, sympathoexcitatory subcortical activity has to be controlled, and research indicated that the PFC—typically associated with governing higher cognitive functions—is involved in regulating activity in sympathoexcitatory subcortical circuits (Thayer et al., 2009). In emotionally stressful situations, the prefrontal cortex disinhibits its inhibitory control over sympathoexcitatory subcortical circuits and lets subcortical neural structures such as the amygdala make autonomic and prepotent responses to situations (Thayer et al., 2009). However, when identifying certain safety signals, the PFC exerts its inhibitory control over sympathoexcitatory subcortical circuits and makes responses that are appropriate for contexts in which the signals occur. Therefore, the inhibitory cortical-subcortical circuit is critical for self-regulation (Thayer and Lane, 2000; 2002). On the other hand, the breakdown of the inhibitory mechanism can result in the constant activation of sympathoexcitatory subcortical circuits, which may lead to emotional, attentional, and autonomic dysreguation and the emergence of perseverative behavior such as worry. Neurochemically, tonic inhibitory control is achieved by γ-aminobutyric acid (GABA) activity within the NTS and reduced GABA activity has been also associated with anxiety, perseverative cognition and poor habituation (Malizia et al., 1998; Friedman, 2007; Thayer and Lane, 2000).

The complex neutral circuits link the inhibitory cortical and subcortical pathways with the heart via the vagus nerve (for reviews, see Benarroch, 1993; Ellis & Thayer, 2010; Thayer et al., 2009). High vagally-mediated HRV indicates an exertion of good cognitive, emotional and physiological self-regulation, which is associated with highly integrated cortical-subcortical circuits (Thayer et al., 2009). In contrast, low HRV is associated with poor regulatory systems resulting from the lack of prefrontal regulation over subcortical activity, which is behaviorally manifested through hypervigilance, the failure to habituate to novel, nonthreatening stimuli, and perseverative behavior such as worry (Friedman, 2007; Thayer et al., 2009).

There exists the relationship between serotonergic activity and HRV. HRV is positively related to serotonin turnover (DePetrillo et al., 1999). Individuals carrying *s* allele of serotonin transporter gene showed reduced HRV compared to *l*-homozygotes (Crişan et al., 2009). Lower levels of serotonin induced by tryptophan depletion were associated with reduced HRV in remitted depressed patients (Booij et al., 2005). Thayer and Ruiz-Padial (2006) suggested that reduced HRV may also indicate the altered coupling or breakdown of the connectivity between the PFC and the amygdala typically exhibited in individuals

carrying *s* allele (Heinz et al., 2005; Pezawas et al., 2005). Increased vagally-mediated HRV was observed after SSRI treatment in panic patients (Tucker et al., 1997) and PTSD (Cohen et al., 2000).

6. Conclusion

In this chapter, we presented potential psychophysiological markers that have been studied in anxiety research. A weak LDAEP may indicate dysfunctional serotonergic activity associated with patients with anxiety disorders. Patients with different subtypes of anxiety disorders may be associated with distinctive LDAEPs and that the LDAEP may serve as a predictor of SSRI treatment outcome in patients with GAD. Also, other ERP components (e.g., P1, P2, N300, P3b, EPN, LPP and ERN) have been useful in studying attentional biases favoring threat-relevant stimuli in highly anxious individuals. Patients with anxiety disorders typically show reduced HRV—indicating autonomic dysfunction caused by elevated sympathetic and reduced vagal cardiac control (Friedman, 2007). Accumulated evidence suggests that reduced HRV—linked with anxiety disorders—may contribute to poor emotional and cognitive self-regulation, the failure of inhibition at multiple levels and perseverative cognition such as worry (Friedman, 2007).

The genetic, cognitive, and psychophysiological characteristics may interact with each other and with other environmental factors such as stress to produce or exacerbate different symptoms in anxiety disorders. Future work may benefit from integrating these markers and exploring the relationships with genetic predispositions to the psychopathology. Of clinical importance is whether these potential psychophysiological markers may play a role in predicting the efficacy of psychological and medical treatments, which is yet to be determined.

7. Acknowledgment

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12

Comorbidity of Anxiety and Affective Disorders as Neuropsychiatric and Evolutionary Problem (A New Concept)

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1. Introduction

Although the comorbidity issues in psychiatry have received a wide recognition for at least the last 20 years and caused a great stream of publications all over the world, many questions remain unanswered. It concerns as phenomenon of comorbidity itself, as plausible explanation of its origin. Little is known, why in some patients the so-called pure (non comorbid) psychopathological states present (i.e. pure depression or anxiety), while in other cases comorbidity between affective and anxiety disorders exists. At present, factors determining the comorbidity between depression and anxiety disorders unfortunately remain unknown.

The principal and unexplained phenomenon in comorbidity issues concern the asymmetry in comorbidity between affective and anxiety disorders (Table 1). Indeed, patients with primary diagnosis of anxiety disorder have more frequent comorbidity with depression (48-62%) than vice versa (10-20%, Table 1). (Kendler et al., 1992; Kessler, 1999; Kessler et al., 1994; 1995; 1996, 1999; Roy-Byrne et al., 2000).

These findings haven't obtained any plausible explanation yet, and comorbidity studies including description of comorbid disorders and statistical evaluation of risk for their development didn't give us any clue (except for genetic origin or predisposing trigger factors) for solving these issues.

On the other hand, neuropsychological or neuropsychiatric mechanisms have been rarely used in order to explain any psychopathological phenomena, and strong demarcation line, unfortunately still exists between neuropsychology and general psychopathology, although the both disciplines represent two domains of neuroscience. It concerns the problems of localization and lateralization of cerebral functions as in so-called normal, as in pathological states, although a great progress has been achieved for the last forty years in realms relevant to these problems. Moreover, epilepsy (especially temporal lobe epilepsy, TLE) seems to represent a useful model for profound understanding of unresolved psychopathological issues, including comorbidity between anxiety and depression.

In the present article an attempt has been made on suggestion one of possible mechanisms for comorbidity development based on neuropsychiatric and evolutionary data, although suggested paradigm, may be criticized and appraised rather as speculative due the lack of direct and strong evidence based data relevant strictly to comorbidity of affective and anxiety disorders.

2. The prevalence of affective and anxiety disorders and asymmetry in their comorbidity

The principal fact should be stressed, that in general population affective disorders in form of depression occur more frequently than anxiety disorders (Table 2).

AD	Prevalence in population	Probability for coincidence with MDD	Comorbidity1 (of MDD with AD)	Comorbidity2 (of AD with MDD)	Ratio of Co-moridity1 to probability for coincidence	Ratio of Co-moridity2 to probability for coincidence
PD	3,4% [2]	0,58%	9,9% [21,24]	55,6% [2]	17,1	95,9
GAD	4 - 6% [44, 45]	0,68-1,02%	17,2% [21,24]	62% [17, 21,22]	16,9 - 25,3	60,8 - 91
PTSD	1,3 -8,4% [45]	0,22- 1,42%	19,5% [21,24]	48% [23,24]	13,7 - 88,6	33,8 - 218

Notes: the lifetime prevalence of MDD according to data by Blazer et al. (1994) consists 16,9%. Comorbidity 1 implies the primary diagnosis of MDD; Comorbidity 2 – the primary diagnosis of AD.

Table 1. Asymmetry in comorbidity between depression and anxiety, lifetime comorbidity of MDD with AD, and lifetime co-morbidity of AD with MDD and calculated variables for co-morbidity probability

Ratios of Comorbidity 1 and Comorbidity 2 to probability for chance expectation show the magnitude of comorbidity level relatively to chance coincidence.
The data on prevalence of affective disorders and anxiety are well
known and have been reported in numerous publications performed mostly by Kessler (1999) and Kessler et al.(1994; 1995; 1996, 1999). The principal data on comorbidity of affective and anxiety disorders are listed in the table 1. This table has been created based on literature findings relevant to prevalence of depression and anxiety and their concomitant existence in general population (Eaton et al., 1994; Kessler, 1999; Kessler et al., 1994, 1995, 1996, 1999; Judd et al., 1998).

Authors	Amount of studied patients	Prevalence of affective disorders (%)	Prevalence of anxiety disorder (%)	Significance of discrepancies
Strine et al., 2005	30018	15,5%	6,8%	p=0,00001
Tellez-Zenteno &Wiebe, 2005	36984	10,8%	4,7%	p=0,00001
Kobau et al., 2006	4151	15%	15%	n.s.
Total	71153	13,8%	8,8%	p=0,00001

Table 2. Comparison of prevalence of affective (depressive) and anxiety disorders in general population

As can be seen from table 1 there are certain discrepancies in variables "Comorbidity 1" and "Comorbidity 2": the "Comorbidity 1" is 2 – 5,5 fold lower, then "Comorbidity 2" level. In other words, the primary and main diagnosis of MDD has caused lower level of comorbidity with any AD, while the primary diagnosis of AD has lead to higher level of comorbidity with MDD, and the last category seems to be more consistently present in comorbid pairs, than AD. These data were firstly revealed by Kessler (1999) and depression itself seems to be more universal and frequent phenomenon in comorbidity than anxiety. At the first glance the cause for such discrepancy remains unclear, although such an asymmetry may be explained partly by more frequent occurrence of pure depressive disorders compared with pure anxiety disorders, and data depicted in tables 2, as a rule, confirm this suggestion.

A genetic loading alone isn't able explain such an asymmetry in comorbidity. If anxiety and depression were regarded as manifestations of one disease with shared genetic inheritance in such a case there could be a symmetry in comorbidity between them and level of comorbidity should approach at least to 1,0 (100%), but this isn't the case.

On the other hand, depression and anxiety can't be regarded as independent phenomena since in this case the comorbidity level should be much lower (as can be seen in second column) than the real data on comorbidity between them.

The question on a temporal sequence in the development of MMD and AD hasn't also received a proper answer, although it seems to be the principal one. Once again we aren't able to explain the real level and asymmetry in comorbidity dependent on main and primary diagnosis. The psychodynamic point of view has been proposed by Wittchen et al. (1994, 2003). According to this scheme, depression develops as secondary disorder to primary anxiety disorder. The last has appeared earlier than former and plays a causal role for depression development. Although such an opinion has become a dominant and widespread, the mentioned model has been obtained mainly on patients with social anxiety and simple phobia, while data on persons with PD or GAD can't be incorporated in aforementioned scheme, because these anxiety disorders are thought to appear in later age (Wittchen et al., 1994; 2003; Baldwin, 2003; Baldwin & Polkinghorn, 2005). It concerns particularly GAD, which age at onset is believed to be at average between 35 and 45 years (Baldwin, 2003; Baldwin & Polkinghorn, 2005). On the other hand, an average age at onset for recurrent depression seems to be younger, and in the Epidemiologic Catchment Area Study has been shown that in subjects who developed a major depressive episode, 20% had initial symptoms by age 19 and subsequently developed a major depressive episode by age 25, while 50% showed depressive features by age 26 and developed depressive episode by age 39 (Rehm et al., 2004). The age of onset for panic disorder, as a rule, coincident with major depression and seems to be in interval between middle to late 20 years (Anderson et al.,1984; Rapee, 1985; Rapee & Barlow, 2004).

Moreover, the so-called simple and social phobia may be regarded as features in the frame of premorbid personality in persons with prevailed anxiety and obsessions. In this context suggestion could be made that such premorbid personality with excessive worries and sense of debt is very similar to *Typus melancholicus* described by Tellenbach (1971). This personality structure is thought to be a risk factor for sequential development of MDD, although not all studies could confirm this rule.

Obviously the mentioned above psychodynamic mechanism alone would not be enough to explain properly the cause of comorbidity between affective and anxiety disorders.

Moreover, such a possible mechanism isn't able again to explain the asymmetry in comorbidity between MDD and AD.
At last, data in last two columns (ratio of Comorbidity1 and Comorbidity 2 to values of probabilities for chance coincidence MDD and respective AD) show the certain discrepancies between these two ratios. Thus, the probability for comorbidity of primary MDD diagnosis with any AD in 14 - 90 times higher, while for comorbidity of any primary AD diagnosis with MDD in 30 -218 times higher, compared with chance expectation for two disorders. Once again the ratio values for Comorbidity 2 are in 3-5 times higher, than for Comorbidity 1. It means that in the occurrence of any AD the presence of MDD presence is much probable, than vice versa. In other words, in the case of primary diagnosis of any AD the appearance of MDD is much more inevitable than in cases of primary diagnosis of MDD, although the plausible explanation for such a discrepancy is still absent. Obviously this can be explained by earlier MDD beginning that precedes the onset of AD, but not vice verse. It concerns strictly the diagnosis of GAD and in less degree the diagnosis of PD, but not the diagnosis of simple and social phobia that begin usually earlier than MDD (Kessler, 1999, Wittchen et al., 2003).
Moreover, the age at onset for MDD after primary beginning of AD is at average 11,2 years higher, than vice versa (i.e. if MDD precedes the onset of AD, as has been shown in National Comorbidity Survey (Kessler, 1999).It concerns mostly for all forms of phobias and Posttraumatic stress disorder, but not PD and GAD (Kessler, 1999). Probably such discrepancy may be explained by more prominent role of genetic factors for primary MDD development, whilst in cases of secondary MDD and primary AD diagnosis the involvement of environmental and psychogenic factors may be more important especially for depression development.

3. The cerebral locus as cause for psychopathology (with emphasis on hemispheric laterality in affective and anxiety disorders)

The data relevant to local cerebral pathology as possible cause for psychopathological signs, unfortunately, are scarce, controversial and have not received yet a proper appraisal. All such findings were obtained, as a rule, on neurologically ill patients, but not in persons with so-called functional disorders, including major depressions and different anxiety disorders. Among neurological diseases more frequently were studied epilepsy, traumatic brain injure, cerebral tumors with psychopathological manifestations, Parkinson's disease, multiple sclerosis and stroke.
In line with main objective of present article the data on affective (depressive) and anxiety symptoms are present without concern other psychopathology.
Numerous data have shown that in patients with stroke the side of damage plays role in depression origin, although all findings remain mostly controversial. Thus, in series of trials, performed by Robinson (2000) and Robinson & Szetela (1981), Robinson et al. (1984) has been shown that in patients with left hemisphere stroke the major depression developed in 60% with lesion in the frontal region, and in 13% with lesion in posterior region. On the contrary, in patients with right hemisphere stroke depression was revealed in 17% lesion in posterior region and in none case in anterior region. It implies that patients with left anterior lesion have significantly higher probability for major depression development than patients with any other lesion location (Robinson, 2000; Robinson et al., 1984).

Moreover, the proximity of lesion to the frontal pole in the left hemisphere occurs to correlate with severity of depression: the closer the lesion to the frontal pole is located the more severe degree of depression is expected. For the right hemisphere strokes such an association hasn't been revealed, and quite the contrary, the closer the lesion is located to posterior pole the more severe depression is observed (Robinson, 2000; Robinson & Szetela, 1981). Such a mirror image relationship between depression and lesion locations seems to point to two different depressive syndromes, which perhaps despite their external similarity, nevertheless, might have hidden discrepancies in their psychopathological symptoms, although this should be proved in a special study.

Moreover, these data fairly consist with Geodakian's paradigm on evolutionary stages of any signs development: old and even ancient signs are affined to posterior brain regions and right hemisphere, while new (recently acquired) signs have affinity to frontal brain regions and left hemisphere (1993).

Another interesting findings, obtained in studies by Robinson (2000) and Starkstein and Robinson (1989) concern the role of family psychiatric history among patients with major depression following right hemisphere stroke. Principally, the similar family loading on psychiatric disorders in patients with left hemisphere lesions hasn't been found (Starkstein and Robinson,1989). This might suggest the different vulnerability for depression development in patients with left and right hemisphere stroke due genetic factors. Patients with depression after right hemisphere stroke seem to be more prone to depression, which in evolutionary terms is older and determined rather more by genetic and less environment factors than depression in patients with left hemisphere stroke, although an external psychopathological similarity should be in both subtypes.

Data on anxiety states due focal brain lesions are less consistent and were observed less frequently. Nevertheless, anxiety is thought to represent the second most prevalent mood disorder after depression in stroke patients, and its prevalence is believed to reach 3,5-24% of all stroke patients (Starkstein et al., 1990; Astrom, 1996). So-called pure anxiety syndromes are thought to occur more frequently in patients with right hemisphere lesions, while anxiety with concomitant depression occurs in persons with left hemisphere strokes (Astrom, 1996; Castillo et al., 1993; Ghika-Schmid & Bogousslavsky, 2000).

Data on interictal anxiety in temporal lobe epilepsy (TLE) are more controversial. Thus, according to data by Dobrochotova & Bragina (1977) depression frequently prevails in patients with right sided foci while in patients with left sided foci, as a rule, more frequently anxiety occurs than depression. Here should be stressed that these data were obtained analyzing the aura semiotics in patients with epilepsy and cerebral tumors, and in this context these data may be not properly comparable with mentioned above findings, since data obtained rather in patients with depression or anxiety states in interictal period are required.

Interestingly, the data by Altshuler et al. (1990) and Perini and Mendius (1984) obtained on patients with TLE in interictal period are consistent with mentioned findings. In their study anxiety disorders were registered more frequently in patients with left than right-foci epilepsy.

Similarly, in study by Kalinin and Polyanskiy (2009) the frequency of diagnosis of organic anxiety disorder in TLE patients with left-sided focus was 3 fold higher than in patients with right-sided focus. On the contrary, in the last subgroup of patients more frequent was the diagnosis of organic affective disorder. In other words, in interictal period in patients with TLE the right focus activity determines mainly affective disorder (depression), while the left focus epileptic activity, conversely, provokes anxiety disorder.

In study by Helmstaedter et al. (2004) the inverse correlations between Beck Depression Inventory score and verbal learning, verbal recognition and figural learning strictly for left lateral temporal lobe epilepsy were obtained, that confirms the significance of foci lateralization for depression development. Interestingly, statistically significant correlations between Depression score and temporal lobe epilepsy with right foci (irrespective of mesial or lateral) and left mesiotemporal lobe epilepsy were absent. It confirms the significance of left lateral temporal foci strictly in the development of depression and cognitive impairment in cases of neocortical, but not mesiotemporal epilepsy, In other words, the depression associated with cognitive impairment origins mostly in persons with neocortical epilepsy and left-sided foci only. Nonetheless, depression can also occur in patients with mesiotemporal epilepsy but irrespective of the foci lateralization, as has been shown in study by Quiske et al. (2000). The authors evaluated the association of MRI-defined mesiotemporal sclerosis (MTS) with Beck Depression scores in 60 patients with temporal lobe epilepsy. Mean depression score was significantly higher in patients with MTS, irrespective of MTS lateralization, and investigators concluded, that depression is a good indicator of MTS, but not vice versa. However in such a case the link between depression and cognitive deterioration is absent (Quiske et al., 2000). It implies that depression may occur irrespective of side foci in paleocortical epilepsy, but predominantly in case of left-sided foci in neocortical epilepsy. Obviously, in evolutionary terms depression in patients with mesiotemporal epilepsy seems to be older compared with depression in persons with neocortical epilepsy.

From evolutionary point of view these findings are consistent with data on depression in stroke patients and stress the significance of left hemisphere lesion (focus) for origin of relatively novel depression combined with cognitive deterioration in evolutionary terms. Nevertheless, the possibility for depression development in patients with right foci (lesions) also couldn't be excluded, but in such a case so – called evolutionary age of depression would be more ancient. In other words, such depression should have earlier age of onset, and probably the lack of cognitive impairment than depression associated with left hemisphere, although this again should be proved in special trials.

In summary, the aforementioned data have showed the possibility for neuropsychological and evolutionary approach at the same time to anxiety and depression evolvement in patients with epilepsy and stroke. Such an approach itself at first glance is not relevant enough to comorbidity problems of affective and anxiety disorders at all. Nonetheless, the possibility exists to extrapolate such approach on comorbidity issues of anxiety and affective disorders too, because certain brain functions *a priori* must be considered as universal for all cerebral disorders and diseases.

4. Asymmetry in neuropsychological tests for verbal and gestalt function in patients with epilepsy

A great deal of data exists that contradict to traditional knowledge of the role the left and right hemispheres in neuropsychological functions processing. Thus, the classical and traditional point of view on distribution of cerebral functions asserts the primary role of left hemisphere in verbal and analytical functions, and right hemisphere in so-called gestalt functions including visual and spatial cognition. Based on this paradigm suggestion can be made, that temporal lobe epilepsy (TLE) with left-sided foci should cause strictly damage of verbal memory and analytical abilities, while in right-foci TLE an impairment in

visuospatial tests should be expected (Lavadas et al., 1979; Mungas et al., 1985; Ossetin,1988).

The findings obtained after selective left- or right temporal lobes had been removed, as a whole, confirmed aforementioned paradigm (Milner, 1965).

This traditional view had steadily existed until the recent years when principally new findings were obtained on patients with TLE. Thus, statistically significant relations between foci lateralization and impairment of verbal and nonverbal memory have been revealed not in all studies (Perrine& Kiolbasa, 1999; Spencer, 1998), that can't be solely explained by traditional paradigm of discrepancies in left- and right hemisphere functions. Proposed explanations usually were based on facts of bilateral temporal lobes involvement in cases of mesiobasal epilepsy (Dupont, 2002; Dupont et al., 2002). In such cases there is no need to contrast left- and right foci in patients with TLE, since hippocampal sclerosis is thought to cause damage as in the left, as in the right temporal lobe.

Nevertheless, the data on differences in capability of left and right hemispheres in neuropsychological test performing by patients with TLE have been observed not only in epilepsy with hippocampal sclerosis, but in cryptogenic epilepsy too.

Thus, in study by Moore and Baker (1996) Wechsler tests for evaluating verbal, visual and general memory were used in patients with TLE resistant to antiepileptic drugs. The patients with left foci performed tests on verbal and logic memory worse, than patients with right foci, as should be expected. Nevertheless statistically significant differences in nonverbal and visual tests between patients with left and right foci haven't been obtained. It implies that nonverbal and visual functions are determined not only by right hemisphere but by left hemisphere too. The similar findings have been also obtained in other studies (Barr& Consortium, 1995; Breier et al., 1996).

Notably, the similar results are observed irrespective of epilepsy form, i.e. as in cryptogenic, as in symptomatic temporal lobe epilepsy (Giovagnoli & Avanzini, 1999). In other words, the brain organic lesion itself, including mesiotemporal sclerosis, isn't enough to explain aforementioned discrepancies in hemisphere functions. The left hemisphere seems to be responsible as for analytic and verbal, as for gestalt function, while the right hemisphere – strictly for gestalt functions. Based on their findings authors concluded that lateralization of brain functions concerns strictly the verbal, but not the visual functions (Giovagnoli & Avanzini, 1999).

Concluding this part of article we may suggest that TLE with left foci is more virulent than with right foci, because it causes deterioration as in verbal, as in nonverbal functions. In this context epilepsy with left foci is quite similar to epilepsy with bilateral foci, and discrimination between them based solely on clinical data seems to be difficult. On the other hand, the right-foci epilepsy seems to be characterized strictly by unilateral damaged functions, determined by the right hemisphere. Any adequate explanations for these discrepancies are still unfortunately absent, but suggestion can be made that part of left hemisphere functions duplicates the properties of right hemisphere in temporal lobe epilepsy. Obviously, an extrapolation from epilepsy model on other pathological states and disorders are probable, since brain mechanisms concerning hemispheric laterality function must be universal.

5. An evolution of brain functions in phylogeny and ontogeny

Ernst Haeckel, a brilliant German biologist and successor of Charles Darwin, had discovered in 19-th century rule, which later has became known under his name as biogenetic law

(Haeckel, 1870). According to this law, the temporal sequence of acquired characteristics in ontogeny is quite similar to that in phylogeny in condensed manner. In other words, the appearance of any signs, symptoms, disorders, and pathology, at all, during individual life repeats its appearance in phylogeny for this species. Based on this rule, the prediction can be made, that earlier appearance of signs and disorders in ontogenesis of *Homo sapiens* reflect their earlier origin in evolution while the appearance of any signs in later period of individual life points out to the novel evolutionary signs.

The further development of Haeckel' law has been made by modern Russian biologist V. Geodakian (1993) , who has extrapolated Haeckel's rule on brain evolution taking into account the role of cerebral lateralization.

In line with Geodakian's rule, the hemispheric lateralization of different cerebral functions also points at different time period for their appearance in phylogeny. Thus, the right hemisphere is responsible for gestalt analysis, and is regarded as ancient brain function, while the left hemisphere, conversely, determines the analytical and verbal processing, that are thought to be the novel acquired brain functions (Geodakian, 1993).

The similar point of view on the cerebral lateralization as indicator of acquired brain functions at different stages of evolution of *Homo sapiens* has been reported by other authors, and particularly by British psychiatrist Timothy Crow (1998).

Taking all these data together, a suggestion can be made, that any characteristics and symptoms in neuropsychiatric disorders associated with left hemisphere should be regarded as younger in comparison with signs and symptoms associated with right hemisphere (Geodakian, 1993; Crow, 1998). In addition, the appearance of right hemisphere signs (i.e. syndromes and disorders) in ontogenesis has to occur earlier than appearance of signs determined by left hemisphere. Nevertheless, in development of certain cerebral disorders the existence of some symptoms and syndromes attributed to left hemisphere is thought to imply the earlier existence of similar signs connected to the right hemisphere, and data discussed above, as a whole, confirm this hypothesis. On the other hand, a definite functions and signs existed that are attributed strictly to left hemisphere (i.e. analytical and verbal functions) but not *vice versa*. In other words, right hemisphere functions seem to be less universal and specific than some functions determined by left hemisphere. If accept that hypothesis, then data on asymmetry in test performance in epileptic patients described in previous chapter can simply be explained: the functions of left hemisphere mean not only verbal and analytical processing, but visual and dimensional too. On the contrary, the functions of right hemisphere seem to be restricted for gestalt analysis. Obviously, the mentioned gestalt functions appear earlier in the right hemisphere and later in the left hemisphere. This explains why a damage of right hemisphere can cause impairment of gestalt processing only, while the damage of left hemisphere leads to impairment of both analytical and verbal, and gestalt processing. At last, if take into account the psychopathological symptoms or syndromes, attributed electively to the left or the right hemisphere, then we may presume that existence of some left hemisphere psychopathological signs imply the existence of similar signs in the right hemisphere in earlier stage of ontogenesis, but not *vice versa*. The left hemisphere seems to be much more functionally capable, than right hemisphere, although this remains a pure speculative suggestion.

Obviously, localization data also should be considered in evolutionary model along with lateralization findings. Here should be reminded a well known scheme, that brain evolution has direction from posterior to anterior regions (i.e. from occipital and parietal lobes to temporal, and from them to frontal lobes). Frontal lobes seem to be the youngest regions of brain in evolutionary terms.

6. Hemispheric and evolutionary hypothesis for comorbidity

In the previous sections of current article an unequal role of left and right hemispheres in certain neuropsychological test has been revealed, and evolutionary approach for explanation in asymmetry in test results has been proposed. As has been shown the left hemisphere is much more capable in test performing (including not only verbal, but gestalt functions too) compared with right hemisphere which functions are restricted entirely to gestalt analysis.

Obviously, the left hemisphere in someway duplicates the functions and properties of right hemisphere (which have earlier origin in ontogenesis) and by that posses more functional variability, than right hemisphere. If accept this hypothesis and extrapolate that to all functions and properties of brain (with emphasis on psychopathological symptoms and syndromes), then data on comorbidity between affective and anxiety disorders may be explained from proposed above point of view.

Really, a certain parallelism between neuropsychological tests performing in epileptic patients and affective and anxiety symptoms development due isolated cerebral hemisphere lesion can be seen, although there are data, that in someway contradict to this paradigm. Nevertheless, depression may been evolved due as the right, as the left hemisphere involvement, and existence of left hemisphere pathology (either depression, or anxiety) implies the previous existence of hemisphere pathology (mostly in the form of depression). In other words, the more variable and broad functions of left hemisphere not only in neuropsychological tests but a greater variability in all properties is seen. It concerns, particularly, affective and anxiety symptoms and syndromes. As has been shown above the left hemisphere is involved in depression and anxiety genesis and by that determines presumably comorbidity between them. Moreover, an asymmetry in comorbidity levels between depression and anxiety may be explained if accept, that pure anxiety syndrome is more attributed to left, than right hemisphere, although this rule has been confirmed only in several studies (Dobrochotova & Bragina, 1977; Altshuler et al. 1990); Perini and Mendius,1984; Kalinin & Polyanskiy, 2009). Conversely, in cases of isolated involvement of the right hemisphere and depression existence the left hemisphere may remain intact. This explains more frequent prevalence of depression over anxiety disorders. All data discussed above also could confirm this suggestion.

Obviously, the existent data are not enough to accept a hypothesis proposed above entirely. Nevertheless, in cases of left hemisphere involvement the concomitant appearance of both depression and anxiety has been observed, that is consistent with mentioned hypothesis.

On the other hand, the mixed and poorly differentiated depressive and anxiety states were registered more frequently in TLE patients with right hemisphere focus. Moreover in such TLE patients the so-called alexithymic features in premorbid period are strongly expressed, and alexithymia complemented to the right-sided focus seems to be a risk factor for depression or anxiety development (Kalinin et al., 2010).

As phenomenon, anxiety is characterized by higher degree of cognitive processing and vigilance in comparison with depression that, in turn, implies the left hemisphere involvement in anxiety disorders. Moreover, traditionally anxiety is thought to be divided on psychic subtype and somatic subtype, and the former is more attributed to left hemisphere, while the somatic anxiety is thought to be attributed to the right hemisphere. Several observations confirm this suggestion. Thus, Reiman et al.(1984, 1986) found PET abnormalities in the right parahippocampal region in patients with panic disorder. Similarly

Fontaine et al. (1990) revealed MRI abnormalities in the right temporal lobe, while Nordahl et al.(1990) reported asymmetric blood flow in the parahippocampal gyrus in patients with panic disorder, attributed to greater increase on the right side. Taking all these data together, conclusion can be made, that involvement of the right temporal lobe or parahippocampal region may be important to the development of panic attacks. Noteworthy, the panic attacks include also symptoms of depression, and are characterized by more pleomorphic psychopathological structure compared with psychic anxiety in GAD.

In conclusion, the current evolutionary and neuropsychiatric approach to comorbidity issue in psychiatry is seen to remain as pure speculative due the lack a necessary amount of evidence based data. Nevertheless, if assumption on unequal frequency of depression and anxiety attributed to right and left hemisphere could be proved, then a rule explaining comorbidity would be formulated: in cases of primary diagnosis of so-called relatively novel in evolutionary terms disorders (attributed to left hemisphere) the greater frequency of comorbid disorder is expected, which is older in evolutionary terms. On the contrary, in cases of primary diagnosis of relatively old disorder (attributed to right hemisphere) the frequency of comorbid novel disorders is probably much less. Certainly, such a rule remains pure speculative hypothesis and must be proved or refuted in special studies in the future.

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13

Pharmacological Resistance of Stress Enhanced Fear Learning in an Animal Model of Post-Traumatic Stress Disorder

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1. Introduction

In anxiety disorders such as PTSD, normal fear responding and learning, which is adaptive and helps us survive, is altered in such way that fear becomes maladaptive, interfering with an organism's ability to alter and adapt behavior in situationally appropriate ways. Maladaptive fear learning is thought to underlie the behavioral symptoms of anxiety disorders such as PTSD (Charney, 2004; Rosen & Schulkin, 1998) when fear and fear responses dominate behavior even in benign circumstances. The fear learning circuit normally participates in adaptive learning and response to danger, however after trauma some individuals show symptoms of PTSD such as: abnormal response to milder stressors, increased vigilance and startle response (American Psychiatric Association., 2000). Stress enhanced fear learning (SEFL) models some specific aspects of PTSD. Using this model we can examine the consequences of trauma--how acute stress or a traumatic event permanently alters the way fear is learned and how these permanent changes in the fear learning circuitry produce maladaptive responses and maladaptive fear learning.

Post-traumatic stress disorder (PTSD) is an anxiety disorder that is debilitating and profoundly affects the lives of men and women worldwide. The Diagnostic and Statistical Manual of Mental Disorders (DSM) criterion for a diagnosis of PTSD requires exposure or experience of a traumatic or life-threatening event (American Psychiatric Association., 2000). Trauma may be caused by combat, violence (such as assault, rape, robbery), severe accidents, disasters (natural or man-made). Any one of these traumatic events will be experienced by one-third of the population (Brunello et al., 2001). While the majority of people will not develop PTSD, it is estimated that 10 to 20% of people who experience an acute traumatic event will develop the disorder (Brunello et al., 2001). Symptoms of PTSD include re-experiencing of the trauma, avoidance, and hyper-arousal. Re-experiencing of the trauma can manifest as vivid and emotionally intense memories of the event in flashbacks, nightmares, or ruminations that give the patient a feeling of re-living the trauma. Avoidance of situations, people, or places that remind patients of the trauma is another aspect of the disorder. Increased physiological and psychological arousal, including enhanced startle response and hyper-vigilance also contribute and are indicative of the maladaptive fear learning associated with PTSD.

PTSD is thought to be much more prevalent than the estimated 7.8%. Furthermore, many cases of PTSD may be unreported and thus undiagnosed (Brunello et al., 2001).

Compounding the difficulty of diagnosis, PTSD is frequently co-morbid with other anxiety disorders, major depression and substance abuse (Goisman et al., 1998; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995), making therapeutic treatment a challenge and in some patients ineffective (Brunello et al., 2001).

Some types of therapy used to treat PTSD include cognitive behavioral therapy (CBT), exposure therapy, systematic desensitization, psychotherapy, and pharmacological therapies. Treatments such as CBT, which include exposure therapy, are a primary treatment for anxiety disorders including PTSD and these therapies can be effective treatments for some features of PTSD, for reviews see (Olatunji, Cisler, & Deacon, 2010; Roberts, Kitchiner, Kenardy, & Bisson, 2010). However, behavioral (exposure) therapy is not entirely effective in eliminating symptoms such as exaggerated responses to milder reminder stimuli (Craske et al., 2008).

Pharmacological treatments for PTSD are also common. Anti-depressant drugs such as selective serotonin reuptake inhibitors (SSRIs) are among the drugs that are frequently prescribed in the initial treatment of PTSD symptoms (Berger et al., 2009). Often medications that have been developed for use in other psychological disorders (depression, anxiety, anti-hypertensives, anti-psychotics) are used to treat PTSD (Hamner, Robert, & Frueh, 2004); however, because the mechanisms of PTSD are not clearly understood pharmacological treatments have also been only partially efficacious. Thus, marginal improvements in PTSD symptoms produced by treatments such as CBT and pharmacotherapy may be because they are not addressing some of the underlying neurobiological changes that lead to some of the maladaptive symptoms of PTSD.

Animal models of PTSD can capture some of the behavioral and neurobiological symptoms of the disorder and are useful as tools to examine specific aspects of maladaptive fear learning that cannot be studied in humans. These models use various methods for simulating a traumatic event; exposure to predator scent, restraint, and Pavlovian fear conditioning that uses tone—foot-shock pairings are among the most common methods. Stress enhanced fear learning (SEFL), however, is an animal model of PTSD that uses un-signaled, unpredictable foot-shock to mimic and induce trauma; using SEFL we examine how new fear learning after trauma is sensitized. The hallmark of SEFL is an exaggerated fear learning to a mildly aversive stimulus (Rau, DeCola, & Fanselow, 2005); the animal learns to fear contextual cues formed in an environment that is distinct and distinguishable from the environment where the trauma took place, but after receiving a single shock responds with exaggerated fear, as if it were in the trauma environment. Thus, in SEFL as in PTSD, fear learning that is normally an adaptive process that helps the animal survive becomes maladaptive and disruptive to normal functioning when inappropriate fear responses are triggered. Using SEFL we can examine two aspects of learning that occur as a result of traumatic stress: the associative component, which is fear learning that is directly conditioned to the contextual cues present during the trauma, and the non-associative component, which is more like sensitization in that new fear is acquired after trauma that is disproportionate and not directly related to the trauma (Ponomarev, Rau, Eger, Harris, & Fanselow, 2010). In the following experiments we examine the efficacy of three compounds on SEFL: midazolam, propranolol, and allopregnanolone.

In Experiment 1 we examined the effects of midazolam, a potent anxiolytic and amnestic agent that readily crosses the blood brain barrier and is a positive modulator at $GABA_A$ receptors. Midazolam is commonly used pre-operatively in hospitals to alleviate patient

anxiety and induce amnesia for pre-anesthesia procedures (Bauer, Dom, Ramirez, & O'Flaherty, 2004). Studies of human memory have found that midazolam induced deficits in declarative memory while sparing implicit memory (Stewart, Buffett-Jerrott, Finley, Wright, & Valois Gomez, 2006; Thomas-Anterion, Koenig, Navez, & Laurent, 1999). In animals, midazolam administered prior to restraint stress reduced later conditional responding in an environment where the animals had received foot-shock (Rodriguez Manzanares, Isoardi, Carrer, & Molina, 2005). However, others studies have found that midazolam altered fear memory in animals that had not experienced prior stress but in animals that had been exposed to prior stress midazolam did not attenuate fear memory (Bustos, Giachero, Maldonado, & Molina, 2010). Thus, in Experiment 1 we investigated how midazolam-induced amnesia for the trauma would impact subsequent fear learning—if memory for the traumatic event is necessary for SEFL to occur.

Experiment 2 examined the effect of systemic administration of propranolol on SEFL. Propranolol is a commonly used heart medication as a treatment for tachycardia and has also been used to treat performance anxiety; it is often referred to as a specific b-adrenergic (Brantigan, Brantigan, & Joseph, 1982; Gerber, Freed, & Nies, 1980) antagonist (although it should be noted that propranolol also influences 5-HT receptors by inhibiting re-uptake of serotonin). Propranolol has more recently been examined as a possible treatment for PTSD (Vaiva et al., 2003). Increased responsiveness of the noradrenergic system in patients with PTSD is thought to underlie some symptoms of the disorder (Southwick et al., 1999) as well as enhance memory formation (Cahill, Prins, Weber, & McGaugh, 1994; Liang, Juler, & McGaugh, 1986). b-adrenergic blockade is thought to dull emotional responsiveness and decrease physiological hyper-arousal (Southwick et al., 1999). Thus, propranolol, because of its pharmacological action at noradrenergic receptors in both the peripheral and central nervous systems is an attractive treatment for symptoms of PTSD. However, the results from experiments in both humans (Kindt, Soeter, & Vervliet, 2009; McGhee et al., 2009; Vaiva et al., 2003; van Stegeren, Everaerd, & Gooren, 2002) and animals (Cahill, Pham, & Setlow, 2000; Muravieva & Alberini, 2010) have been inconsistent. In Experiment 2 we wanted first to determine if peri-trauma (administration of the drug both before and after trauma) injections of propranolol would mitigate SEFL, and second to determine if propranolol had an effect upon the memory for the trauma.

In Experiment 3 we examined how acute administration of the neurosteroid allopregnanolone (3-a-hydroxy-5 a -pregnan-20-one) would affect SEFL. Allopregnanolone is a powerful positive allosteric modulator of $GABA_A$ receptors containing the a4 and d subunits (Smith, Shen, Gong, & Zhou, 2007). Thus, allopregnanolone enhances GABAergic transmission, increasing inhibitory influence of the neurons resulting in anxiolysis, sedation, and analgesia (Belelli & Lambert, 2005). Evidence from functional magnetic resonance imaging (fMRI) studies show that the amygdala, which is known to be important in fear learning, recognition and expression, was observed to be over-active in patients with PTSD (Rauch, Shin, & Phelps, 2006; Rauch et al., 2000). GABAergic disinhibition can produce increases in neural excitability and plasticity that may lead to pathological anxiety (Rainnie et al., 2004; Shekhar, Truitt, Rainnie, & Sajdyk, 2005), and levels of allopregnanolone in plasma as well as in the brain are increased in response to acute stress. These increases in allopregnanolone are thought to act as a regulatory mechanism that restores balance between excitatory and inhibitory neurotransmission after stress (Barbaccia, Concas, Serra, & Biggio, 1998; Purdy, Morrow, Moore, & Paul, 1991). In patients with PTSD, decreased

levels of allopregnanolone in cerebrospinal fluid have been observed and severity of symptoms was correlated with low levels of allopregnanolone (Rasmusson et al., 2006). In mice that experienced social isolation stress and enhanced contextual fear conditioning, structures involved in fear learning (hippocampus, amygdala, and frontal cortex) showed decreased levels of in allopregnanolone (Pibiri, Nelson, Guidotti, Costa, & Pinna, 2008). Systemic injections of either allopregnanolone or S-norfluoxetine mitigated the enhanced contextual conditioning, presumably by exerting their effects at $GABA_A$ receptors containing the a4, a6 and d subunits where neurosteroids have high affinity (Pibiri et al., 2008; Pinna, 2010). Changes in gene expression in the SEFL phenotype reveal down-regulation of several $GABA_A$ receptor subunits, among the down-regulated receptor subunit types are the a4 subunits (Ponomarev et al., 2010). Therefore, we boosted circulating levels of allopregnanolone during traumatic stress or during the single shock trial in order to examine if allopregnanolone could mitigate trauma induced over-activation in the brain by enhancing GABAergic transmission globally, thus decreasing SEFL.

2. Method

2.1 Subjects

In the following experiments subjects were experimentally naïve adult male Long-Evans rats purchased from Harlan (Indianapolis, IN). At the beginning of the experiment rats were 90 days old and weighed 340-370g. Rats were housed individually on a 12-hour light/dark cycle with free access to food and water. Animals were initially handled 1-2 minutes per day for a week prior to the start of Experiment 1. Procedures used in these experiments were conducted in accordance with the Division of Laboratory Animal Medicine and approved by the Animal Research Committee at the University of California, Los Angeles. For Experiments 1 and 2, 32 rats were used for each experiment; in Experiment 3, 40 rats were used.

2.2 Apparatus

2.2.1 Experiment 1

Procedures took place in two distinct training/testing environments, Context A, the trauma environment and Context B, the novel environment. Each context contained distinguishable background noise, lighting, and odor in fear conditioning boxes that differed in interior size/shape, texture and grid floor pattern designed to minimize generalization between the contexts. Context A chambers (28x21x22 cm; Lafayette Instruments, Lafayette, IN) were aluminum sided with an opaque Plexiglas back and clear Plexiglas front door piece. Grid floors (18 stainless steel rods, 4 mm diameter, 1.5 cm apart) were connected to a shock generator and scrambler (Lafayette Instrument Co.; Lafayette, IN). Overhead fluorescent lighting and a ventilation fan provided 70 dB of background noise. Fear conditioning boxes were cleaned and dried between each session using a 10% sodium hydroxide solution. Stainless steel pans were placed beneath each grid floor in the chambers, these contained 4-5 sprays of atomized Simple Green as the context odor. Metal scaffolding attached to a cart was used to transport the animals in their home cages to Context A and back.

The interior of the Context B chambers, initially the same as described above, were modified by inserting white Plexiglas along the rear wall and two white Plexiglas side-walls at 60^o angles that formed an A-frame. As in Context A, the front door piece consisted of a clear Plexiglas panel. The grid floor, connected to a shock generator and scrambler (Lafayette

Instrument Co.; Lafayette, IN), consisted of 17 stainless steel rods (4 mm diameter) spaced in offset rows 1 cm vertically and 2.6 cm horizontally. Lighting consisted of one red 30-watt incandescent bulb. A white noise generator produced background noise (70 dB, A-scale). Fear conditioning chambers were cleaned, using a 1% acetic acid solution, and dried between each session. Stainless steel pans were placed beneath each grid floor in the chambers; these contained 4-5 sprays of an atomized 11% coconut extract mixture as the context odor. Animals were transported to and from their home cages in a covered, black rubberized box subdivided into four areas by black Plexiglas panels.

2.2.2 Experiments 2 and 3

Behavioral testing took place in fear conditioning chambers (30x25x25 cm, Med-Associates Inc., St. Albans, VT), which were equipped with a Med-Associates VideoFreeze system and sound attenuating chambers. The SEFL 15-shock trauma procedure was conducted in a set of four conditioning chambers (Trauma context); the single shock and SEFL memory test were conducted in a separate set of four conditioning chambers (Novel context). The two contexts were housed in separate rooms and were perceptually distinct from one another, differing in interior chamber shape, room and chamber lighting, scent, cleaning solution, background noise, and transport to the conditioning chambers.

The Trauma context interior was rectangular in shape with aluminum side-walls, a Plexiglas rear wall either with blue dots or covered with an opaque white panel, and a clear Plexiglas hinged front door panel. The grid floors that deliver the electric foot-shocks consisted of evenly spaced standard grid rods (4.8 mm thick, Contextual Conditioning System, Med-Associates Inc.). Chambers received light from sources mounted above each chamber; the room that housed the conditioning chambers was a standard overhead fluorescent light. Stainless steel pans were placed beneath each grid floor in the chambers; these contained 4-5 sprays of atomized Simple Green as the context scent. Fans mounted on each conditioning chamber provided background noise (60 dB). Animals were transported in their home cages using a portable cart affixed with hanging racks and covered by a white sheet. Between groups of animals chambers were cleaned with 70% isopropyl alcohol.

For the Novel context, where the single shock and SEFL memory test took place, black plastic inserts were used to create a triangular shape (60^{o} angles that formed an A-frame) within the conditioning chamber. The rear of the chamber was covered with an opaque white panel, and the front, hinged door was clear Plexiglas. The grid floors consisted of vertically and horizontally off set rods (4.8 mm thick, Contextual Conditioning System, Med-Associates Inc.). The black plastic inserts obscured the top lighting within the conditioning chambers, and the room lighting consisted of one red 30-watt incandescent bulb. Stainless steel scent pans contained 4-5 sprays of atomized 1% acetic acid solution as the context scent. The background fan in these chambers was turned off (<50 dB). Animals were transported to and from their home cages in a covered, black rubberized box subdivided into four areas by black Plexiglas panels partially filled with rodent bedding; the subdivided box was transported atop a small rolling cart. Between groups the chambers were cleaned with 1% acetic acid solution. All chambers were cleaned with a 10% bleach solution at the end of each day of testing.

2.3 SEFL procedure and extinction

Animals were placed into fear conditioning chambers where they received 15 (1 mA, 1 sec) foot-shocks; control animals received equivalent context exposure but without foot-shock.

The foot-shocks were un-signaled, inescapable and occurred at pseudo random intervals temporally spaced from three to eight minutes apart for a period of 90 minutes. Twenty-four hours later, animals were placed into a novel context. As described above odor, lighting, spatial location, enclosure shape, flooring, and background noise were unique to context (set of four conditioning chambers). In the Novel Context, baseline activity levels and fear were assessed for the first three minutes. Following the initial three minutes the animals were given a single (1 mA, 1 sec) foot-shock and level of fear was measured for five minutes. Animals were then returned to their home-cages. The test of SEFL occurred 24 hours later when the rats were returned to the context where they received the single shock; the animals were then given an eight-minute test of contextual fear learning. Control animals received the same amount of exposure to the trauma environment but did not receive foot-shocks. On subsequent days, the control animals received the same treatment as the SEFL group, a single shock in the novel environment and a test of fear to that second environment.
In Experiment 1, extinction took place in the 15-shock trauma context. Animals were placed into the conditioning chambers for five 30-minute context extinction sessions spaced 5-minutes apart. Exctinction procedures occurred on the day following the 15-shock SEFL procedure and 24 hours before the single shock in the Novel Context.

2.4 Systemic injections

Animals were habituated to positioning and handling associated with i.p. injections for 2-3 days prior to the beginning of each experiment.

2.4.1 Experiment 1

On the drug injection day midazolam animals were injected using 29-gauge 0.5 cc sterile insulin syringes. Animals received injections of either 2 mg/kg of midazolam hydrochloride (Hospira, Inc., Lake Forest, IL, USA) at a concentration of 5 mg/mL or the equivalent amount of sterile saline (0.9%). Injections occurred 20 minutes prior to the start of the 15-shock SEFL procedure.

2.4.2 Experiment 2

Animals were injected with either DL-propranolol (Sigma-Aldrich, St. Louis, Mo., USA), dissolved in phosphate buffered saline (PBS) for a final concentration of 5mg/ml, or PBS with 26-gauge sterile syringes. Animals were given injections (5 mg/kg) both 20 minutes prior to the 15-shock SEFL conditioning and within 20 minutes following the SEFL procedure; thus, animals received either a total of 10 mg/kg of propranolol or equivalent volumes of PBS over two injections. At this dosage, studies have found that locomotor and exploratory activity was unaffected, but did reduced the effects of predator stress on behavioral measures of anxiety such as social interaction, hole board, light/dark box, elevated plus maze, and after re-activation of memory in an inhibitory avoidance procedure (Adamec, Muir, Grimes, & Pearcey, 2007; Przybyslawski, Roullet, & Sara, 1999).

2.4.3 Experiment 3

Animals were given i.p. injections (26-gauge sterile syringes) of either allopregnanolone (ALLO, Sigma-Aldrich, St. Louis,
Mo., USA) suspended in a 22% solution of (2-hydroxypropyl)-b-cyclodextrin solution (CDX) in sterile saline (0.9%) for a final concentration of 3 mg/ml, a 22% solution of CDX in sterile

saline (0.9%), or injections of sterile saline (0.9%). Animals were given injections 20 minutes prior to the 15-shock SEFL conditioning; 5 mg/kg of ALLO and equivalent volumes of CDX solution or Saline. Using this dose of ALLO studies found behavioral effects on alcohol consumption after injections but no adverse side effects in rats (Janak & Gill, 2003; Janak, Redfern, & Samson, 1998) and in mice doses of 5-10 mg/kg or greater are used (Pibiri et al., 2008).

2.5 Designs and analysis

2.5.1 Experiment 1: Midazolam

Animals were randomly assigned to one of three groups: midazolam pre-trauma (MDZ/Trauma), n = 12; saline pre-trauma (SAL/Trauma), n = 12; or saline no-trauma (SAL/No Trauma), n = 12. On day 1 all animals received systemic (i.p.) injections of either midazolam or saline followed 20 minutes later by the 15-shock SEFL procedure. Twenty-four hours later, on day 2 all animals were extinguished in the trauma context (Context A) using a massed extinction procedure. On day 3 animals received the single shock in the Novel context and were tested for new fear memory in that context 24 hours later (day 4). Fear memory for the 15-shock trauma context was measured on day 5. For the trauma context fear memory test 2 animals per group were not used due to an experimenter error yielding the following group sizes: midazolam pre-trauma (MDZ/Trauma), n = 10; saline pre-trauma (SAL/Trauma), n = 10; or saline no-trauma (SAL/No Trauma), n = 10.

Data Analysis

Freezing scores were used as a measure of learned fear (Fanselow, 1980), and were determined by averaging over behavioral observations (recorded on video tape) made every 8 s during each test (percentages were calculated by dividing number of observations of freezing by the total number of observations multiplied by 100). Freezing is defined as the lack of movement except for respiration (Fanselow, 1980). Freezing scores were calculated for baseline freezing in Context B prior to the single shock, post-shock freezing in context B, freezing conditioned by the single shock in Context B, and freezing in the original trauma context (A). Conditioning and testing were videotaped and a trained, blind observer scored and calculated freezing scores.

Freezing scores were calculated as described above and group differences were analyzed using a one-way analysis of variance (ANOVA) for each designated time period. Statistical significance was determined at $p < 0.05$. Post-hoc multiple comparisons among groups were made using Tukey HSD tests in order to specify group differences within each measurement period.

2.5.2 Experiment 2: Propranolol

Animals were randomly assigned to one of four groups in a 2x2 factorial design. Animals were designated by prior experience as having either received either No Prior Trauma or Trauma, and by drug treatment either injected with propranolol or vehicle. Thus the four groups consisted of: No Prior Trauma/Vehicle, n = 8; No Prior Trauma/Propranolol, n =8 ; Trauma/Vehicle, n = 8; Trauma/Propranolol, n = 8. On day 1 of the experiment all animals were injected systemically (i.p.) with either propranolol or Vehicle 20 minutes before the start of the 15-shock SEFL procedure. Following the SEFL procedure animals were re-injected with propranolol or vehicle. Twenty-four hours later, on day 2, all animals were placed in the Novel context where they received a single foot-shock. On day 3 animals were

tested for SEFL during a fear memory test in the Novel context. Animals were placed again in the trauma context, where they had received the 15 shocks and were tested for fear memory on day 4.

Data Analysis

Freezing scores were used as a measure of learned fear (Fanselow, 1980; Fanselow & Kim, 1994). Freezing behavior was scored using VideoFreeze software (Med-Associates Inc.). During behavioral testing, digital video information from cameras mounted in conditioning chambers was recorded in near-infrared light (NIR). An analysis algorithm that compared pixels of a sample reference frame against real-time continuous frame samples was summed to produce the Motion Index. Activity below a Motion Index of 50 units for duration of 30 frames (or more) per second was scored as freezing. In a validation study, freezing scores generated by the VideoFreeze software and freezing scores generated by human scoring were highly correlated, $r = 0.971$, $p < 0.0001$ (Anagnostaras et al., 2010); a high correlation between the VideoFreeze scoring and two highly trained observers ($r > 0.9$) has also been reported by this laboratory (Jacobs, Cushman, & Fanselow, 2010). Average freezing scores were calculated for baseline freezing in the novel context for 3 minutes prior to the single shock; post-shock freezing in the novel context was assessed for 5 minutes. The SEFL test in the novel context and the trauma context fear memory test measured freezing for 8 minutes. Freezing scores were calculated as described above and group differences were analyzed using a two-way analysis of variance (ANOVA) for each designated time period. Statistical significance was determined at $p < 0.05$.

2.5.3 Experiment 3: Allopregnanolone

Animals were randomly assigned to one of five groups. All animals experienced the same behavioral procedures: 15-shock trauma, a single shock in the Novel Context, the SEFL test in the Novel Context, and a fear memory test in the Trauma Context. Animals received either ALLO, saline, or CDX vehicle prior to the 15-shock trauma, or ALLO or saline prior to the single shock in the Novel Context. The five groups were as follows: ALLO Pre-trauma, n = 10; SAL Pre-trauma, n = 10; CDX Pre-trauma, n = 8; ALLO Pre-1shk, n = 6; SAL Pre-1shk, n = 6.

Data Analysis

Data analysis was similar to data analysis described for Experiment 2, however for Experiment 3 a one-way ANOVA was used to determine statistical significance among groups.

3. Results

3.1 Experiment 1: Midazolam alters fear memory for trauma but does not alter SEFL

Novel Context baseline fear

As Figure 1 illustrates all three groups showed very low levels of fear to the Novel context during the 3 minute period before the onset of the single shock. There were no statistically significant differences among the groups, $F(2, 33) = 1.38$, $p = 0.27$. Lack of group differences in baseline fear indicates whatever fear was acquired during the trauma experience did not generalize to the Novel context.

Novel Context post-single shock fear

Fear in the Novel context during the 5 minute period following the single shock was low in the SAL/No Trauma animals while post-shock freezing for both the SAL/Trauma and MDZ/Trauma groups was high. Figure 1 illustrates these group differences. A one-way ANOVA confirmed these group differences, $F(2, 33) = 11.98$, $p < 0.001$, and post-hoc comparisons revealed significant differences between the SAL/No Trauma group ($M = 14.17$, $SD = 14.67$) and both prior trauma groups, however there was no reliable difference between SAL/Trauma animals ($M = 63.54$, $SD = 35.82$) and MDZ/Trauma animals ($M = 43.26$, $SD = 32.99$).

Novel Context: SEFL memory test

Fear memory for the Novel Context was measured to assess the amount of context specific fear was acquired by the single shock on the previous day. The measure of freezing during the 8 min 32 s test serves as the primary indicator of stress enhanced fear learning. Figure 2 demonstrates this effect; animals that received no prior trauma show a small amount of new fear to the single shock context while animals that received the prior 15-shock trauma display a disproportionately high amount of new fear, that is enhanced fear learning, was unmitigated by experiencing trauma under the drug midazolam. These observations were bourn out by a one-way ANOVA, $F(2, 33) = 6.33$, $p < 0.01$. Post-hoc analyses confirmed that the SAL/No trauma group ($M = 9.25$, $SD = 11.26$) differed from both the SAL/Trauma ($M = 38.93$, $SD = 24.72$) and the MDZ/Trauma groups ($M = 36.33$, $SD = 28.28$). There was no reliable difference in freezing between the SAL/Trauma and MDZ/Trauma groups.

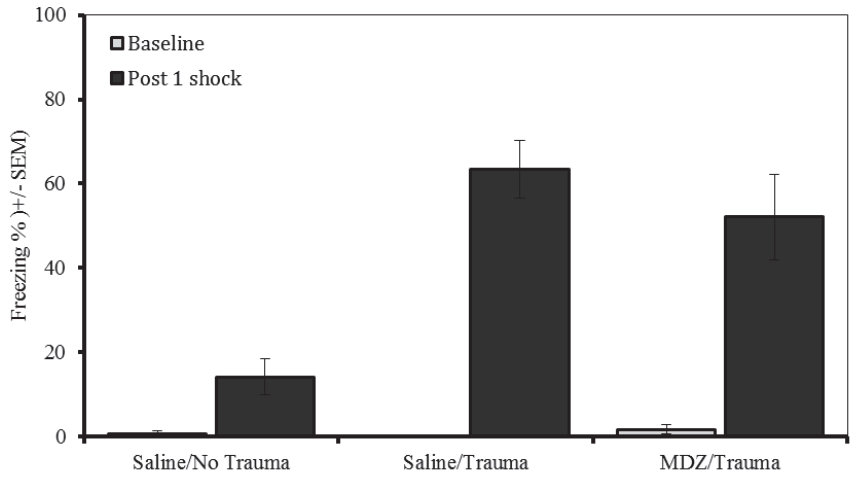

Fig. 1. Experiment 1 Systemic midazolam: Novel Context baseline and post-single shock fear. Mean percent time freezing (+/- SEM) in the Novel context before and after the single shock. All groups showed very low freezing during the baseline measurement period preceding the single shock. Following the single shock animals that did not have prior trauma showed little fear after the single shock, whereas both the saline and midazolam groups that had prior trauma showed high levels of fear following the single shock.

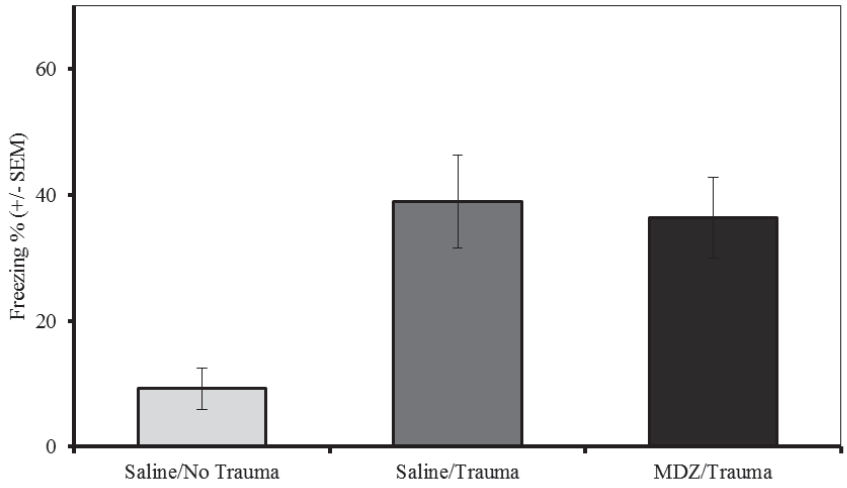

Fig. 2. Experiment 1 Systemic midazolam: *Novel Context SEFL memory test.*
Mean percent time freezing (+/- SEM) in the novel context 24-hours after the single shock. The saline injected group without prior trauma showed low to moderate levels of fear in the Novel context. The saline and midazolam injected groups that had prior trauma showed equivalent levels of enhanced fear, indicating that midazolam did not attenuate SEFL.

Trauma Context memory test

Fear memory for the Trauma context was assessed during an 8 min 32 s period on the day following the SEFL test in the Novel Context. As shown in Figure 3 animals that did not receive the prior trauma in this context showed low levels of freezing; animals that received saline injections before the 15-shock trauma displayed comparatively high levels of freezing, while animals that received midazolam before the 15-shock trauma showed a decrease in fear to the trauma context. A one-way ANOVA revealed reliable differences among the groups, $F(2, 27) = 3.70$ $p < 0.05$. Post-hoc analyses confirmed differences between the SAL/No Trauma group ($M = 8.13$, $SD = 8.52$) and the SAL/Trauma group ($M = 32.03$, $SD = 19.66$), but no reliable difference from the MDZ/Trauma group ($M = 25.31$, $SD = 27.81$). The lack of reliable difference between the MDZ/Trauma group and the SAL/No Trauma group suggests that midazolam altered memory for the traumatic experience; however, the MDZ/Trauma group was also not reliably different from the SAL/Trauma group, suggesting partial amnesia for the trauma.

The findings from Experiment 1 suggest that SEFL is unchanged by manipulations that alter the fear memory for the trauma; neither partial amnesia produced by midazolam in conjunction with massed extinction, nor massed extinction alone were able to diminish SEFL. This is consistent with the findings of Rau et al. (2005) in which neither spaced extinction nor disruption of context fear learning were able to abolish SEFL; spaced extinction over five days, and intra-ventricular administration of the N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-amino-5-phosphonovalerate (APV) effectively eliminated fear in the trauma context, yet SEFL still occurred. Thus, even while the associative

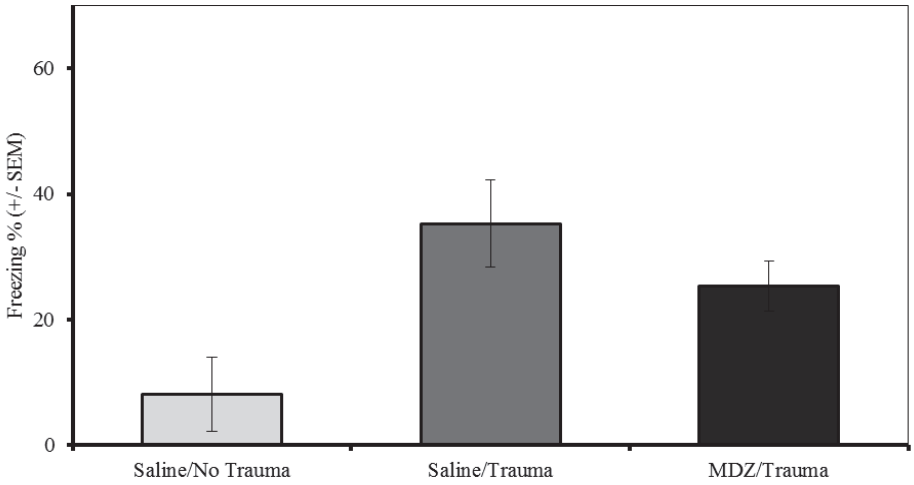

Fig. 3. Experiment 1 Systemic midazolam: Trauma Context memory test.
Mean percent time freezing (+/- SEM) in the Trauma context. Saline injected animals that did not have prior trauma displayed little fear for the trauma context while saline injected animals that did receive the 15-shock trauma displayed fear even after extinction of fear in this context. Midazolam injected animals showed some fear memory for the trauma context, however, compared to saline/trauma counterparts fear memory was diminished.

component of SEFL, the fear directly related to the trauma, can be altered and diminished by various methods, the non-associative sensitization of fear learning remains unaffected. Because both extinction and manipulations of memory for the trauma are exerting their effects upon the associative aspects of trauma-related fear, in Experiment 2 we used propranolol, which has been found to reduce memory enhancements for emotionally arousing material (Cahill, Babinsky, Markowitsch, & McGaugh, 1995) and has been studied as a possible treatment of PTSD (Vaiva et al., 2003).

3.2 Experiment 2: Propranolol does not diminish SEFL or alter trauma fear memory

Novel Context SEFL memory test

As illustrated in Figure 4 animals that received no prior trauma under vehicle or propranolol showed low levels of fear when tested in the Novel environment where they had previously received the single shock, whereas animals that had experienced the 15-shock trauma either with vehicle or propranolol showed high levels of fear. These group differences were confirmed statistically; a two-way ANOVA revealed a main effect of prior experience (No prior trauma or Trauma), $F(1, 28) = 40.51$, $p < 0.001$. There was no main effect of drug and no interaction. These results indicate that propranolol had no effect on the acquisition of new fear in the single shock environment—SEFL was evident in animals that had received prior trauma whether they had received vehicle or propranolol.

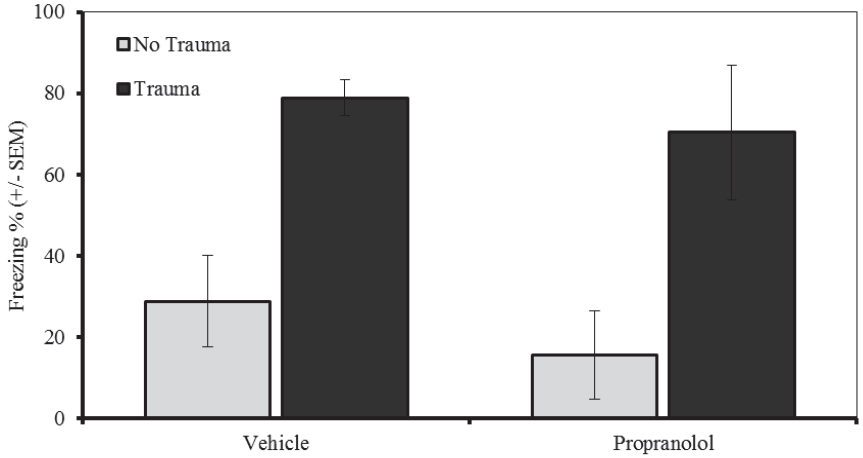

Fig. 4. Experiment 2 Systemic propranolol: *Novel Context SEFL memory test.*
Mean percent time freezing (+/- SEM) in the Novel context 24 hours after the single shock. Animals that had no trauma and received either vehicle or propranolol did not differ in fear learning to the single shock context. Vehicle treated animals that had prior trauma showed the typical SEFL phenomenon. Propranolol treated animals with prior trauma also showed SEFL, indicating that propranolol administration did not alter subsequent fear learning.

Trauma Context fear memory test

Animals displayed the same pattern of results as in the SEFL test; regardless of whether animals were treated with vehicle or propranolol, animals that had not received prior trauma showed low levels of fear when returned to the trauma context and animals that had experienced trauma there showed very high levels of fear. This is depicted in Figure 5. Statistical analysis confirmed that there was again a main effect of prior experience (No prior Trauma or Trauma), $F(1, 28) = 72.40$, $p < 0.001$, but no main effect of drug and no interaction. The clear absence of an effect of propranolol suggests that the drug was not producing behavioral changes that should be evident if the drug had altered how the trauma had been experienced.

We found that propranolol, when administered systemically before and after traumatic stress, had no effect on SEFL, nor did it alter fear memory of the traumatic event. Thus, neither the associative nor the non-associative aspects of SEFL were changed by propranolol. The null results of propranolol that we observed may indeed be similar to the findings of McGhee et al. (2009) who found no effect of propranolol on the development of symptoms of PTSD in soldiers who suffered combat related burns. However, these findings are in contrast to Viava et al. (2003) who found that instances of PTSD were reduced when propranolol was given post-trauma to car accident or assault victims. Acute stress is known to engage stress response centrally as well as peripherally. One biological effect of stress in the brain, and a consequence of corticosterone (in rats) release, is endogenous release of the neurosteroid allopregnanolone. Allopregnanolone is co-released from GABAergic neurons and is a positive modulator at $GABA_A$ receptors, enhancing GABAergic transmission in the brain. Allopregnanolone is produced and released centrally as well as peripherally, crossing

the blood-brain barrier readily. Thus, in Experiment 3 we examined the effect of boosting levels of available allopregnanolone via acute systemic injections either before the traumatic stress or before the single shock episode.

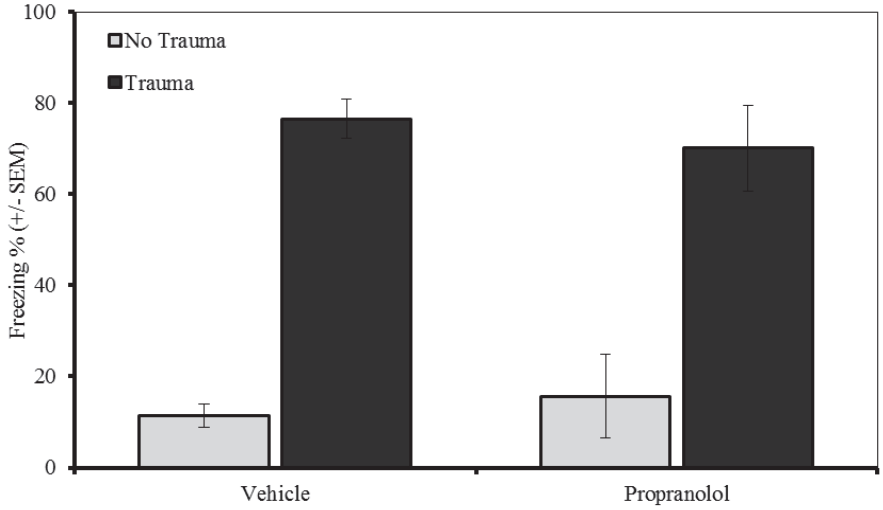

Fig. 5. Experiment 2 Systemic propranolol: *Trauma Context fear memory test.*
Mean percent time freezing (+/- SEM) in the 15 shock Trauma context. Both vehicle and propranolol injected animals that did not have prior trauma displayed low levels of freezing. Similarly, vehicle and propranolol injected animals that had prior trauma showed high levels of freezing, suggesting that fear memory for the trauma context was not altered by propranolol.

3.3 Experiment 3: Systemic administration of allopregnanolone does not alter SEFL

Novel Context SEFL memory test

As illustrated in Figure 6 animals that received ALLO administration prior to the trauma or prior to the single shock showed no reduction in SEFL, and animals in both the saline injection and CDX control groups displayed characteristically high levels of fear in the Novel Context. A one-way ANOVA confirmed that there were no overall group differences, $F(4, 35) = 1.60$, $p = 0.196$. These results suggest that the anxiolytic properties of allopregnanolone were not sufficient at this dose or by this route of administration to mitigate the fear sensitizing effects of prior trauma.

Trauma Context fear memory test

Figure 7 shows the same pattern of results as seen in the SEFL memory test. ALLO did not attenuate associative fear learning in the Trauma Context. As expected, SAL and CDX controls showed very high levels of fear when returned to the Trauma Context. Group differences were not statistically significant, $F(4, 35) = 1.51$, $p = 0.22$. Fear memory for the Trauma environment was not decreased by prior administration of allopregnanolone, suggesting the effect of systemic allopregnanolone had no detectable effects on the 15-shock trauma.

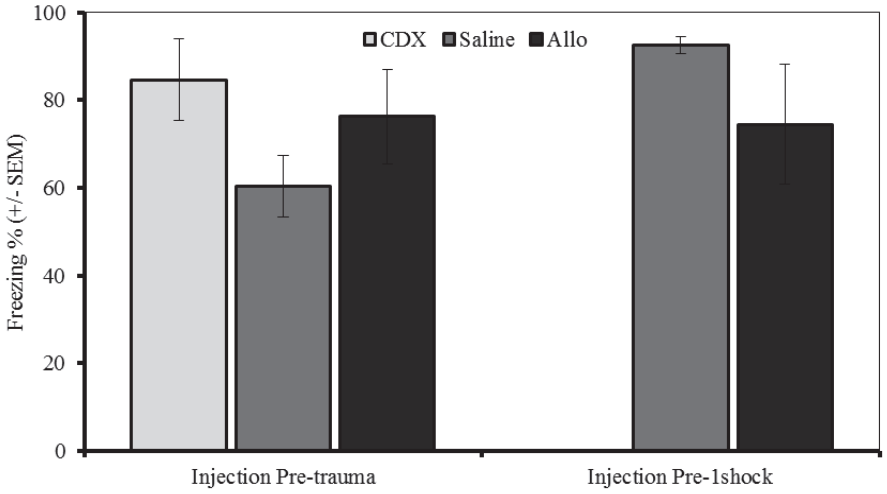

Fig. 6. Experiment 3 Systemic allopregnanolone: *Novel Context SEFL memory test*.
Mean percent time freezing (+/- SEM) in the Novel context 24 hours after the single shock. Pre-trauma injections of allopregnanolone, CDX, or saline did not reliably diminish exaggerated freezing in the Novel context. In animals that received injections of either saline or allopregnanolone before the single shock there were also no reliable differences.

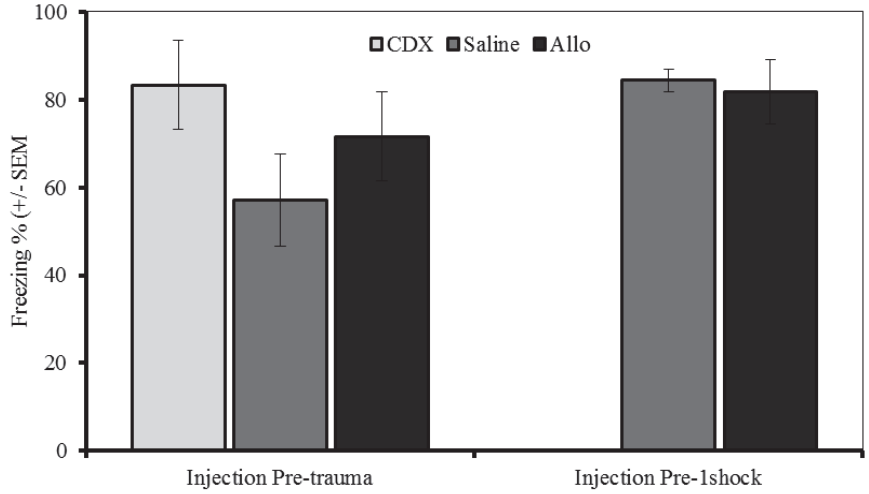

Fig. 7. Experiment 3 Systemic allopregnanolone: *Trauma Context fear memory test*.
Mean percent time freezing (+/- SEM) in the 15 shock Trauma context. Trauma context fear memory was not reliably altered by pre-trauma injections of allopregnanolone, CDX, or saline. Fear memory for the trauma context was not changed by subsequent administration of either saline or allopregnanolone.

Our results from Experiment 3 indicate that allopregnanolone given in an acute systemic does not attenuate SEFL at the dose of 5 mg/kg. We found that systemic injections of the drug administered either before the traumatic event or before the single shock stressor had no effect on how new fear was acquired subsequent to trauma. These findings are in contrast with those of Pibiri et al. (1998) and Pinna (2010) who found that systemic administration of allopregnanolone reduced negative consequences, such as enhanced contextual fear conditioning, in rats that had experienced social isolation stress.

4. Discussion

Our results indicate that SEFL is resistant to pharmacological manipulations that targeted either the GABAergic or noradrenergic neurotransmitter systems. Midazolam, though it appeared to alter the memory for the traumatic event, was ineffective at eliminating SEFL. Indeed, neither midazolam nor allopregnanolone, both of which increase GABAergic inhibition (achieved via potentiation of somewhat different $GABA_A$ receptor populations) affected SEFL. Nor was SEFL affected by manipulation of noradrenergic transmission using the b-noradrenergic blocker propranolol.

SEFL is a robust behavioral phenomenon that seems to be refractory to several of the pharmacological treatments used to treat anxiety and PTSD; indeed, the SEFL inducing trauma seems to produce behavioral symptoms akin to treatment refractory PTSD where the symptoms of the disorder are not mitigated by behavioral and pharmacological treatments. Additionally, systemic administration of drugs, while useful for clinical translation, is a limited tool in that we cannot specify where in the brain the drug will exert an effect. Indeed, global effects may reflect contradictory actions of the drug; among brain regions it is possible that the lack of drug effects may occur by offsetting actions on different brain regions. While the three compounds that we examined in these experiments all readily cross the blood brain barrier, we should not overlook possible effects of the drugs in the periphery or overlook the role of feedback from the periphery influencing processes in the central nervous system. In these experiments we used acute administrations of each drug on a single day of the experiment. Although the time points for drug administration were chosen in order to assess specific effects upon either the trauma memory or upon single shock memory it is possible that multiple doses following the trauma (more similar to pharmaco-treatment regimes) might have produced a detectable effect.

Limitations of midazolam

Although midazolam, administered systemically, was likely acting upon diverse regions in the brain, it is believed that the effect of the drug is mainly exerted in the hippocampus (Frank, O'Reilly, & Curran, 2006). The effect of midazolam on inhibition of neurons within the hippocampus explains midazolam's amnestic quality, GABAergic transmission there would be increased, potentially deterring the formation of declarative memory. Rau et al. (2005) has previously shown that amnesia for the trauma could be induced using intra-ventricular infusions of APV to block NMDA receptors primarily in the dorsal hippocampus. Thus, memory for the trauma is not necessary to produce SEFL; the associative learning about the trauma can be blocked while the non-associative processes that lead to SEFL are left unchanged. The findings reported here are similar to those reported by Rau, Fanselow, and Eger in (Sher & Vilens, 2010); when the SEFL test occurred after a five day period between the trauma/drug administration midazolam did not

diminish SEFL but did alter memory for the trauma. Rau et al. used a 4 foot-shock procedure, which has previously been shown to produce SEFL almost as robustly as the 15 foot-shock procedure (Rau, Oh, Laster, Eger, & Fanselow, 2009), and although in the current experiments we used the more severe 15 foot-shock procedure, midazolam was still able to diminish the associative memory of the trauma. The dose used in the present experiments was slightly higher than in the Rau et al. experiments (2 mg/kg vs. 1.5 mg/kg), however the 15 shock procedure lasts 90 minutes while the 4 shock procedure lasts only 20 minutes. It is possible that midazolam induced only partial amnesia for the trauma context (rather than the abolition of fear observed in the Rau et al., experiments) because of the length of the conditioning sessions; during the latter portion of the 15 shock procedure the midazolam could have been less effective, allowing contextual fear learning in the trauma context to occur.

Limitations of propranolol

It is possible that time of treatment may be a factor that determines the efficacy of propranolol. Early studies using propranolol were especially exciting because it could be administered after the occurrence of trauma. This is important given the unexpected nature of trauma, and rather than requiring prophylactic administration *in case* trauma occurs, propranolol could be given to victims of trauma following the event. However, because we administered the drug both prior to and following the trauma we were able to take advantage of propranolol's effect on general arousal (decreasing heart rate and blood pressure (Gerber et al., 1980)) as well as its reported post-trauma effects on emotional memory.

The second difference among studies is the type of trauma: in the human literature, when propranolol was tested for efficacy after combat-related trauma, propranolol was ineffective (McGhee et al., 2009) while propranolol administered after car accidents or assault was found to be effective. We speculate that the 15-shock trauma may have been more similar to the intensity of trauma produced by a combat situation, and that the severity of the trauma is possibly a factor in propranolol's efficacy. Propranolol may be effective in mitigating certain types of trauma while for other types the effect of propranolol may not be powerful enough to ameliorate the effects of severe trauma. There is some evidence that propranolol may be an effective in altering fear memory during re-consolidation (Kindt et al., 2009). However, human laboratory fear learning procedures are far less severe than either exposure to real trauma in humans or foot-shock trauma in rodents. The possibility remains that reconsolidation of learned fear might be altered by the physiological effects of the drug; memories of the trauma that are recalled under propranolol might produce less physiological arousal, decreasing emotional salience. However, SEFL is unchanged by extinction as well as amnesia for the trauma (Rau et al., 2005) and along with our findings in Experiment 1 this suggests that manipulations focused on altering the associative component of SEFL may not be effective.

A third possible difference among the outcomes of propranolol studies is the type of learning or memory that is being evaluated, and what symptoms or behaviors are being measured. Emotional memory per se can be studied in rodents, by measuring freezing to assess fear learning and expression; however in human studies self-report measures or galvanic skin response are often employed. Additionally, studies of emotional learning and memory often focus on associative symptoms directly related to the traumatic event; however, SEFL is a phenomenon that illustrates a non-associative processes and behavior

that is indicative of a sensitization of fear learning—most studies do not look at these processes. These non-associative processes, however, are likely to be significant contributors to PTSD symptomology.

Emotionally arousing stimuli may increase noradrenergic activity and thereby enhance memory (Cahill et al., 1994; Roozendaal, Okuda, de Quervain, & McGaugh, 2006), but this effect may be limited to milder emotionally arousing situations. It is possible that in our findings we did not detect any diminution of enhanced memory in the propranolol group because of the strong emotional effect produced by the 15-shock trauma. Perhaps if the trauma procedure was less emotionally arousing it might be possible to discern an effect of propranolol. Reist et al. (2001) found that propranolol had the same effect of reducing recall for an emotionally arousing story in patients with PTSD as for control subjects, suggesting that propranolol might be effective at reducing memory for subsequent milder stressors that lead to behavioral phenomena like SEFL. However, this suggests that propranolol would not be an effective treatment for the most severe aspects of PTSD, which are those most in need of treatment.

Paradoxically, extreme arousal of the NE system causes memory deficits (Kobori, Hu, & Dash, 2010). In this regard it is possible that propranolol was actually having a dulling effect on the trauma memory so that the lowered NE arousal allowed memory formation about the trauma when it might otherwise have been decreased. However, given the data from the control groups, which show identical levels of trauma context fear, the animals without propranolol were able to learn and remember the trauma context as well as their propranolol counterparts.

Limitations of allopregnanolone

Allopregnanolone has been shown to produce behavioral changes in other paradigms such as in operant conditioning experiments looking at voluntary alcohol consumption. Behavioral effects at doses of allopregnanolone that were similar or even lower than the dose used in Experiment 3 have been observed (Janak & Gill, 2003; Janak et al., 1998); doses of 3 mg/kg and 5.6 mg/kg increased alcohol consumption, suggesting that the reinforcing properties of alcohol, exerting its effects at the $GABA_A$ receptors containing a4 subunits, was enhanced with the administration of allopregnanolone.

There is evidence that tonic GABAergic inhibition via interneurons in the basolateral amygdala are responsible for modulation of excitatory projection neurons in this region (Ehrlich et al., 2009), and that GABAergic disinhibition produces increases in neural excitability and plasticity leading to pathological anxiety (Rainnie et al., 2004; Shekhar et al., 2005). We had hypothesized that by elevating circulating levels of allopregnanolone before trauma that the neurosteroid might be able to enhance GABAergic response to the traumatic stress, thereby impeding over-activation of brain regions involved in fear learning and the consequent long-term effects of trauma. A single acute administration of allopregnanolone, however, may not have a strong enough influence on the fear circuitry to counteract the effects of trauma. SEFL is a long-lasting behavioral effect that has been observed as long as 90 days after the initial trauma (Rau 2009). Thus, the trauma seems to be permanently altering the fear learning circuit and it is likely that a transient boost in allopregnanolone was not able to mitigate the effects of trauma.

Down-regulation the $GABA_A$ receptor a4 subunit gene as a consequence of SEFL (Ponomarev et al., 2010) led us to speculate that during stress allopregnanolone at the a4-containing receptors might act as a protective mechanism. However, allopregnanolone did

not alter SEFL or fear memory for the trauma. Since we did not measure direct changes in $GABA_A$ receptor a4 subunit expression after acute administration of allopregnanolone under conditions of traumatic stress and because SEFL behavior was unaffected, we can only speculate that down-regulated expression of the $GABA_A$ receptors was not affected by allopregnanolone. Likewise, in the animals that received allopregnanolone on the day following the 15-shock trauma and before the single shock in the novel environment we saw no effect. In this case it is possible that because changes in receptor expression can occur very rapidly, that down-regulation of the $GABA_A$ receptor a4 subunit had already occurred as a consequence of trauma, and furthermore that by the time the allopregnanolone was administered there were fewer receptor targets, especially in the amygdala, at which to exert an effect. A final concern about the use of allopregnanolone is that allosteric modulators often act as feedback mechanisms in the brain. Thus, a surplus of allopregnanolone might have interfered with endogenous feedback mechanisms, producing contradictory effects to those of endogenous allopregnanolone release.

5. Conclusion

Many of the pharmacological treatments for anxiety disorders such as PTSD focus on ameliorating symptoms that are specifically related to the fear learning and memory of the trauma. These types of treatments, though they may be effective treatments for cognitive and declarative aspects of the disorder, do not affect symptoms like SEFL, which may be responsible for some of the treatment refractory symptoms of PTSD.

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Anxiety Disorders in Dogs

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1. Introduction

Dogs have been one of the closest domesticated animals to men who live with them, and in the majority of cases bond in such a way that they represent another family member, reason why their behaviour affects the common welfare directly. Changes in dogs' behaviour represent a serious problem that threatens not only the physical integrity and general well-being of the dog but also that of the people around it. This way, studies focused on resolving animal behaviour problems are indirectly helping in enhancing the family's life quality and even the community's; likewise they reduce the pet's home exclusion risk, since the majority of dog behaviour problems usually lead into the dog's sacrifice or abandonment. All of these situations frequently cause anxiety problems in humans.

Anxiety is a common reason of consulting in Animal Psychiatry. Of all the treated patients in the Service of Clinical Ethology of the Veterinary Faculty of Madrid, an approximate 88% showed anxiety-related disorders. Studies made by other authors also show numbers similar to ours which leads us to say that the majority of dog behaviour disorders are accompanied by anxiety. This is the reason why they are considered an important problem in the behaviour veterinary medicine.

The majority of behaviour problems related to anxiety or fear that can be found in dogs fit in the normal adaption answers categories, being considered abnormal in the situations that represent a conflict with the animal's environment. It's normal for dogs to respond with fear to some stimuli that are unknown to them and to which they weren't introduced. It's normal to show aggressive behaviour when confronting an individual that is perceived as a threat and from whom there is no escape, and to use aggression more quickly in these situations as a defence mechanism.

2. Etiology

The presence of a significant change in the animal's life or those situations capable of producing chronic or post-traumatic stress, result in the alteration of the animal's homeostasis and an adaptation disorder, causes of anxiety disorders (Bousoño et al., 1999; Brousset et al., 2005). There are several factors that predispose towards anxiety, such as genetic factors and experiences during development and learning. The knowledge and study of these elements is essential for the correct diagnosis and application of the ideal therapy. On the other hand, aside from the intrinsic factors, stimuli coming from the

environment or the lack of said stimuli belong to the realm of the acquired and, are more influential during the early stages of life; likewise, the lack of control and prediction of the environment also act as influential factors. These imbalances are responsible of the apparition of a psychological conflict, which produces anxious responses, which limits the individual to co-exist in equilibrium with its environment, specially resulting into a social malfunction.

3. Anxiety disorders

Several definitions for anxiety disorders have been proposed, all of them depending on the different authors' interpretations. In general, it is accepted that the majority of behaviour disorders are related to anxiety. Overall (1997) stipulates a link between stress and the apparition of anxiety. On the other hand, anxiety is the apprehensive anticipation of a stimulus or situation that the animal perceives as unpredictable or dangerous, adopting a preparation and answer behaviour towards the situation or stimulus that might occur (Beata y col., 2006).
Anxiety is pathological when it is continued or grows in an endogenous way without environmental conditions justifying it, becoming uncontrollable by the dog. This type of anxiety present in pathologies such as phobia, separation anxiety and many ways of handling fear, make the dog enter a self-stimulation spiral which enables it to reach a state of tranquillity and homeostasis. This sort of anxiety requires pharmacological treatment; otherwise, it could result into the worsening of the behaviours with which it is associated, and even depression.
The most frequent anxiety-related problems are: separation anxiety, generalized anxiety, aggressiveness, fears, phobias and obsessive-compulsive disorders.

3.1 Separation anxiety

Dog's separation anxiety is defined as the behavioural disorder which appears when a dog is left on its own at home or when it is separated from its owners (Sherman and Mills, 2008). It's common in individuals that haven't done a correct detachment, a particular period in their development around the time of puberty where they see that the braking of the affective bond is made by the mother. Signs of separation anxiety in dogs are shown in the situations in which the subject is unable to be with the owner or with the person to whom they are attached (Pageat, 2000).
Separation anxiety may be related to stress signs caused by situations, more frequent in time, such as the dogs being left alone for long time intervals because of the owners' normal habit, or because of restrictions in the social interactions with other dogs, exploratory behaviour and physical exercise (Sherman and Mills, 2008). Likewise, the lack of a physical and social environment for the dog causes a state of boredom and frustration, which can become accumulative and generate separation anxiety.
Symptoms of this disorder frequently appear when the animal perceives that it is about to be left alone, being more obvious when the owner goes out, and becoming more intense between 30 and 60 minutes after leaving. These behaviours were described and numbered by Overall (1997), under which we find excessive vocalization (increase in whining, howling and barking), destructive behaviour (especially directed at objects frequently manipulated by the owners' and that carry their smell), restlessness (manifested as an exacerbated exploratory behaviour), inappropriate defecation and urination, hyper-salivation and escape

attempts. However, not all dogs that come to the clinic with this disorder show all of these symptoms, it's not only possible to see one, two or all of them at the same time, but also other more uncommon symptoms such as anorexia, vomits and acral dermatitis due to licking. All of these behaviour changes are accompanied by a stress indicative physiological answer. Dogs with separation anxiety are described by their owners as "very clingy", it's common that they follow one or more family members step by step; trying to go with them into all of the house's rooms. This tends to intensify itself near the owner's departure. It is also typical that owners describe their dogs' welcoming as an excessive effusive reaction (Manteca, 2003).

3.1.1 Etiology

Normally, 2-3 month old puppies are separated from their mother while still being strongly attached to them, which can cause a tranquilizing effect and a fundamental support for them to develop all of their behaviours. When the separation happens, the pup suffers an ill-state which can manifest itself with whining during the night and the lack of appetite, from which the animal develops an adaptive behaviour in search for a balance, establishing a new bond of attachment with one of the owners, this being very positive for the puppy because it allows it to finish its development. Under natural circumstances it is the mother that systematically begins this rupture, which in many occasions doesn't happen in the bond created by the puppy and his owner. The owner continues to respond to the dog's demands, which has already reached puberty, this provokes a hyper-attachment that is nothing but affective dependence, which will be the origin of separation anxiety (Pageat, 2000).

In our clinic we have been able to see that there are situations that frequently result into a separation anxiety disorder, such as those where the dogs, being used to a continuous human company are left alone for the first time as well as the situations during which the dog and owner are constantly together during a long period of time (because of vacation, sick leave or unemployment). Likewise, separation anxiety can arise after the dog has suffered a traumatic event, such as a time period spent in a shelter or dog pound, or after a change in the routine or family structure (child emancipation, modification in the working schedule, moving to a new home, or a new pet or person in the house). Nevertheless, although it is important to take them in consideration when making a diagnosis or when preventing, separation anxiety does not necessarily present itself after one of these situations. Within the possible factors, genetic predisposition has also been mentioned; however it has not been demonstrated.

3.1.2 Diagnosis

The clinical record should be done via questionnaire that can provide all the possible information about the dog's daily routine related with food, defecation and urination habits, properties of the physical surrounding, social interaction (with other animals as well as with people from their surroundings), physical exercise and the dog's rest. All of this information is focused on obtaining a better knowledge about the dog-owner relation and to evaluate if the dog has, qualitative and quantitatively speaking, all of its basic needs covered. Questions that might add information about the dog's behaviour prior to the owner's departure should be considered as well as the circumstances under which such anxious behaviours are triggered should be determined precisely. It is also important to know the dog's attitude when the owners return home. Horwtiz (2006) says that the behaviour's time

sequence is important to establish a diagnosis, there are studies that affirm that the typical separation anxiety behaviour presents itself between 5 and 30 minutes after the dog is on its own at home (Borchelt and Voigh, 1982).

It can be suspected that a dog suffers from separation anxiety when it shows one or more of the following symptoms: destructive behaviour, inappropriate elimination and excessive vocalization (Scopelliti and Bracchi, 2000). Dogs that have separation anxiety with a high level of owner dependence show each of the followings symptoms:

- A tendency to follow the owner through out the whole house seeking to maintain a constant physical contact.
- The increase of physical distance with the owner triggers some anxious conducts in the animal.
- When the owners return home the dog shows excessive enthusiasm.

Scopelliti and Bracchi (2000), indicate that from the category of dogs with separation anxiety, the following dogs can be excluded:

- Dogs younger than 6 months
- Dogs that do not show destructive behaviour or that if they do, they do it in front of the owners.
- Spontaneous episodes.
- Destructive behaviour or aggressiveness-related vocalization
- Inappropriate elimination behaviour (urine) linked with territorial marking in males or with the inappropriate elimination related to physical illnesses.

3.1.3 Differential diagnosis

According to the symptoms, the differential diagnosis proposed by Horwitz (2006) for the separation anxiety firstly includes for destructive behaviour cases: destruction as if playing, hyperactivity, lack of physical exercise, storm and noise phobias, territorial behaviour and fear. When there are signs of excessive vocalization, playing, fear or phobia; external stimuli and territorial behaviours should be considered. Inappropriate elimination is a symptom that should discard: fear, improper training, inaccessibility to the place of elimination, physical sicknesses, submission urination, urine marking and cognitive dysfunction. When there are self-traumatizing behaviours, they should be differentiated from acral dermatitis because of licking, obsessive-compulsive disorder, dermatological problems and neuritis.

On the other hand, (Pageat, 2000) indicates that the deprivation syndrome, sociopathies (dominant dogs tend to stop the departure of other members of the group to which they belong), hyper-attachment of the adult syndrome and involution depression should be discarded.

3.1.4 Treatment

The efficient treatment of separation anxiety should include teaching the dog how to tolerate the owner's absence and correcting the specific problems of destruction, barking and elimination (Landsberg et al., 1998). It is vital to convince the owner of the need to break the existing hyper-bond and to teach and involve said owner in the fulfilment of the therapy. Treatment must include environmental enrichment to reduce anxiety symptoms and minimize the tension present at home, which will allow the therapy to work. Any possibility that the dog might respond with anxious signs must be eliminated, thus leaving the dog alone must be avoided; in case that it should happen, it should be done in a safe place where

the dog won't hurt itself or destroy any objects. Activities that boost the dog-owner interaction such as physical exercise and games must be indicated to increase the dog's wellbeing, as well as reinforce with food treats any wished behaviour (Sherman and Mills, 2008).

3.1.5 Pharmacology

It is necessary to control anxiety with anxiolytic drugs to encourage the behaviour modification therapy and to achieve short-term improvements that will motivate the owner in completing the treatment (see dosage table and anxiolytics' indications for dogs). Better results have been obtained for the treatment of separation anxiety when combining a behaviour modification program and a pharmacological treatment, rather than just using the behaviour modification program (Landsberg et al., 2008; Simpson et al., 2007). The aim is to reduce anxiety and fear as fast as possible as to establish an appropriate emotional balance in the dog so that it may respond better to the behaviour modification program (Sherman and Mills, 2008). The use of a benzodiazepine for four weeks, combined with fluoxetine (a selective inhibitor of the serotonin reuptake) has show to be efficient in controlling the signs of anxiety (Ibáñez and Anzola, 2010).

3.1.6 Behaviour modification techniques

The goals of behaviour modification techniques, desensitization and counterconditioning, that are used in the separation anxiety treatment tend to diminish the anxiety that is associated with the owner's departure, reduce the over-attachment and hyper-link between the owner and the dog and teach the dog how to remain home alone, without anxiety (Horwitz, 2006).

Behaviour modification tries to avoid that the dog feels anxious and that it may remain tranquil while the owners return home. It must comprehend that the owners always return once they have left: for this, the dog must be given the opportunity and all the time that it may require. The anxiety that is produced during this time interferes with the learning and so it is necessary to propose an ansiolytic pharmacological treatment that will favour the establishment of the animal's cognitive abilities. It is very important that during this first period of treatment, the dog should not be left alone at home; the owner will have to provisionally resolve this problem by finding a place other than the home for the dog and offer the necessary means so that it does not miss his company.

Firstly, the owner must have control over his pet. To increase the owner's control over the animal, he will practice basic obedience exercises such as the commands of sit and stay. It is also necessary to practice relaxation techniques through massage routines and conditioning using treats every time the dog controls its anxiety, this way behaviour opposite to anxiety is reinforced. Once this first step is achieved, a technique based on learning by desensitization is begun, to get the animal to accept or tolerate the owner's departure from home. To obtain this, a progressive form of departure is programmed which will allow the animal to predict, through the signals that it receives, the owner's exit. Systematic desensitization is a technique that has been successfully used in humans and pets. There are recent studies (Botler et al., 2001) that claim that systematic desensitization is a key and fundamental element in the treatment of dogs with separation anxiety.

Guideline to follow:

- Begin the normal routine departing activities (get dressed, grab the keys, etc.), make the dog get used to said stimuli. The owner must proceed calmly and ignoring all of the

dog's responses, sitting in a relaxed way without leaving the house. Once a more relaxed attitude from the dog has been achieved, the owner can move on to the following step.

- Begin as indicated in step one. However, instead of sitting down, the owner should head towards the exit door, open it without leaving, then closing it and sitting down. This must be repeated various times through out the day, until checking that the animal responds with complete tranquillity.
- The following step consists on repeating the previous two, but this time remaining outside for a moment without moving or entering, then sitting calmly on the sofa. Repeat until it is assured that the dog tolerates the situation.
- Next, leave and close the door for a few seconds, then open it, enter the house and sit again on the sofa, trying to accustom the dog in tolerating short term absences, starting with a few seconds. The routine will be repeated and the dog will be given a signal like "later", leaving and coming back in a minute. The return must be made as something normal, ignoring the dog.

Proceed gradually from one step to the other and before taking on the following, repeat until the dog shows no sign of anxiety. All the possible ways of leaving home that last less than 10 minutes must be practiced. Many departures can be made per session if the dog manages to relax enough in between them. Once the dog can accept short-term outings (30 to 60 minutes), it will normally be able to tolerate longer intervals of time, between 3 and 8 hours. However, at the beginning the steps should be taken slowly.

3.2 Generalized anxiety

In generalized anxiety the animal shows a constant and crescent reactivity, alertness and exploration, and a great motor activity that interferes with a normal social interaction. When anxiety is constant, it continuously alters the individual's behaviour and it manifests itself as an inhibited stated associated with the production of substitutive activities (Pageat, 2000). When the environment lacks stimuli or they are few, the described signs appear very frequently, without the need of the existence of a triggering stimulus.

3.2.1 Diagnosis

Generalized anxiety is shown as a disorder where a constant exhibition and growing hyper-reactivity, motor activity, alertness and exploration are shown. Like in other anxiety-related disorders, the main symptoms may vary, frequently being nervousness, trembling, muscular tension and palpitations between others. These signs may present themselves under the complete absence of a triggering stimulus. According to what has been established for diagnostic criteria DSM-IV and CIE-10 for generalized anxiety in humans, it is state that manifests anxiousness in a persistent and infinite way, under no predominant environmental circumstance in particular. Generalized anxiety is not exclusive to human beings, having similar behaviours in other animals such as dogs been seen (Overall, 1997).

As a diagnosis reference we can use the information given by Diez (1991) who grouped generalized anxiety symptoms in four conceptual units, which help understand them better, and can be applied in dog's generalized anxiety in accordance to our criteria, since we can easily observe some of the signs or signals described below:

1. Apprehensive expectation. The patient feels apprehensive, generally preoccupied and ruminative. It anticipates that something bad will happen to him (fainting, lose control

or dying) or to the people around him (sicknesses, accidents). Inner restlessness appears the feeling of threats, vague fears, insecurity, sensation of being empty, feeling of nothingness and dissolution of the self.

2. Motor tension. It refers to finding the patient trembling, restless, startled, shivering, tense, subject to muscular pain, easily fatigable and incapable of relaxing. Frequent blinking, tense brow and face, unstable pace, hyperactivity, nervousness and restlessness are also detected. The underlying common characteristic is an increased striated muscular tone. Behavioural manifestations can go from extreme excitement to stuporous inhibition, in extreme and infrequent cases.
3. Autonomic hyperactivity. Palpitations, respiratory fatigue, nausea, thamuria, dizziness, sweating, abdominal pains, trembling and cold and wet skin. To this we would have to add mydriasis, vasoconstriction, diarrhea and chest tightness.
4. Alertness and Scrutiny. Anxious expectation can be seen as "sentinel behaviour". The patient is nervous, impatient and irritable. The subject is alert, hyper vigilant, has movement difficulty, insomnia, interrupted sleep and evident fatigue when waking up.

In a dog's case it isn't possible to know if it has any premonitory thoughts, however, an apprehensive attitude is obvious in the majority of the cases that are diagnosed with generalized anxiety and that come to the clinic.

3.2.2 Treatment

3.2.2.1 Behaviour modification techniques

Counterconditioning and desensitization techniques are the most used in the treatment of anxious dogs. Through obedience and learning techniques, the dog is taught how to show a new adaptive behaviour in presence of the stimulus that causes the anxiety, and that is completely incompatible to the undesired behaviour. The first thing the dog must learn is to show the substitutive behaviour under situations that do not trigger the anxious response and later respond in the same way under the triggering stimulus' presence. This exercise will be done gradually, allowing the animal to elaborate an adaptive strategy. The stimulus exposure time as well as the presentation degree must grow gradually while the animal gets used to it or becomes impervious to it and said wanted behavior must always be reinforced with a treat (Overall, 1997). A detailed protocol must be designed for each particular case, with all the exercises to be made so that the owner may work in a precise way and that it may be used to analyse the animal's progress in the following weeks.

3.2.2.2 Pharmacological treatment

In these cases pharmacological treatment is necessary since anxiolytic medication facilitates the learning process of behaviour modification techniques in dogs. The treatment with ansiolytics should last between 30 to 60 days, once the animal has acquired a normal behaviour or achieved a great improvement; the dosage will start being gradually reduced during 10 or 14 days, to prevent collateral effects caused by the abrupt interruption of the therapy (Overall, 1997). See table of ansiolytics administration, dosage and indications for dogs.

3.3 Aggressiveness

Aggressiveness has been identified as one of the most frequent problems in dogs. A study carried out in Canada concluded that an approximate 15% of dogs, out of more than 3000, had bitten a family member at some point. In the United States alone approximately 2

million people are bitten by dogs every year and it is the cause of 10-16 deaths, this makes canine aggression an important problem for public health as well as a public danger (Manteca, 2003). In other countries including Spain there isn't a significant difference with the described data. This is why it is necessary that the owners of aggressive dogs urgently evaluate what degree of danger their dogs represent and that they find professional counselling to correct mentioned above problem (Landsberg, 2003). These statistics could fall short if we considered that aggressiveness in dogs is occasionally a desired behaviour for those owners that have wanted to train them as guard and defence animals. In this sense, Overall (1997) indicates that some animal owners consider their pets heroes, rather than potentially aggressive dogs, when having been aggressively defended by them from attacks or other people's threats in the street or at home.

Pageat (2000) defines aggressiveness as a reactive state characterized by a higher probability of triggering an aggression. The aggressive subject reacts more often than others, producing aggressions. Aggression includes a great variety of behaviours from subtle gestures or corporal postures and facial expressions, to explosive attacks (Landsberg, 2003).

As per Mertens (2006), the dog learns how to be aggressive in order to achieve a goal, this happens when the aggression allows the dog to achieve control of the situation, and the learnt response is even more intense when rewarded either by petting, talking, or through certain gestures and postures from the owner towards the dog. Overall (1997), compares aggression in dogs with diabetes, stating that neither is curable, but controllable thanks to a well established diagnosis and a correctly applied therapy. It has been proven that there is a link between low concentrations of serotonin in the brain and spine and an increase in aggressiveness (Brown et al., 1979).

Pageat (2000) defends that patients with anxiety become irritable and can easily develop aggressiveness. Other authors such as Reisner (2006) also linked anxiety and aggressiveness when explaining that dogs that bite their owners, even inside a social context, their motivation can be based almost entirely on the anxiety that they are suffering. Some types of aggressive behaviours have been reduced in frequency and intensity with the use of psychotropic drugs that increase serotonin levels (Fuller, 1996; Oliver et al., 1995). The American Psychiatric Association doesn't consider aggressiveness in humans a separate diagnostic category; aggression is the main problem in a great variety of psychiatric disorders, which, according to Dodman and Shuster (1998) also apply in veterinary medicine.

3.3.1 Etiology

The development of an **aggressive** behaviour in dogs is a complicated and multifactor process. The expression of the behaviour can be influenced by many factors, such as the perinatal ones that include the extra uterine environment and the interactions with the mother and the siblings. Experiences acquired during the learning and socialization period, as well as other biological factors also influence in aggressiveness (Haug, 2008). On the other hand, a medical problem can increase irritability which could later trigger an aggressiveness disorder or worsen the already existing aggressiveness problem. This is why, it is essential to discard any possible medical cause that might provoke or catalyse aggressive behaviour, such as, amongst others, hepatic problems, intracranial neoplasm, cerebral hypoxia, endocrine disorders, infectious sicknesses (for example, rabies, canine distemper), disorders in the animal's development (hydrocephaly), intoxications (due to metals,

organophosphates), apoplexy attacks, traumas or other causes that might cause pain, or an increase in the prolactine concentration induced by drugs, or during a false pregnancy (Landsberg, 2003, Mertens, 2006).

The manifestation of an aggressive behaviour may be influenced by the environment, a determined situation or because of certain people's presence. In this sense, we have mentioned that learning is an important factor in the development of an aggressive behaviour. Mertens (2006) explains that basically, the dog learns to be aggressive in order to achieve a goal, following the principal of instrumental conditioning and adds that any daily encounter of the dog with people, dogs or other animals can produce an important impact in the development of unwanted behaviours, including aggressiveness. Many situations that involve the owner may result in aggressiveness related conflicts, such as those that can happen during walks when the dog is tied up or loose or wondering around without supervision. Likewise, barking at the pedestrians through the house's window or during the time the dog spends in the fenced garden. In these cases the owner tends to reinforce the aggressive behaviour with the behaviour he uses in the previously mentioned situations such as touching, petting, pushing or throwing, talking (appeasing or by verbal reprimands) and through gestures and postures that include visual contact, corporal postures and emotional responses.

3.3.2 Types of aggressiveness in dogs

3.3.2.1 Dominance aggressiveness

Dominance aggressiveness is one of the most frequent dog aggressions towards people (Manteca, 2003). It is more frequent in males and in those animals that have reached puberty; signs may be seen at age 3 or less (Landsberg, 2003). Depending on the motivation, there can be two types of dominance aggressiveness which can receive two different denominations: competitive dominance aggression and aggression related to social status or the hierarchy in the man-dog relation (Mertens 2006). In the competitive form, the aggression is frequently done in those situations where a resource has enough value as to fight for it. Pageat (2000) considers aggressiveness to be related with social status, or a hierarchic conflict, such as an alteration in the social relationship between man and dog which can present itself in a vague context, which is denominated sociopathy and that affects the social group and not the individual. On the other hand, Overall (1997) indicates that dominance aggressiveness is the expression of a complex multifactor disorder that is influenced by the social context and the animal's anxiety level.

3.3.2.1.1 Diagnosis

It is diagnosed through the animal's history or the direct observation of its interaction with its owner, where directed aggression towards the owner can be observed in situations where the highest spot of the hierarchy, assumed by the dog, is threatened. This disorder is made of two very well differentiated elements: aggressive behaviour and dominance exhibition**.** This is why, the diagnosis isn't only obtained based on aggressions directed towards the family, but also on the dominance indicating corporal attitudes that the dog shows (ears pulled forward, high tail, fixed staring, trying to be physically on top of the family members). It is also possible that the animal demands being petted or that it stops members of the family from accessing certain places of the house (Landsberg, 2003).

Because of its relation with anxiety, the manifestations of dominance aggressiveness are extremely variable, from aggressive behaviour demonstrations which are well defined and

that are associated to a clear affirmation of control, to very subtle vocal expressions. The difference is how the dog perceives and uses the information obtained from the encounter. The motivation for aggression may vary depending on the context or situation in which the encounter happens, for example a famished dog may attack when it assumes that another individual may stop its access to food, as well as other dogs that react aggressively if a person tries to take away their favourite resting spot; in other cases they compete over toys or for the owner's attention (Overall, 1997). Sociopathies are alterations that appear in the context of an ambiguous relation, which means we won't diagnose this pathology in dogs that completely dominate their owners, but in those animals whose hierarchic situation is vague due to the attribution of prerogatives that are usually associated to the status of being dominant, while the owner takes on a dominant position in hierarchy significant situations. As a consequence signals such as aggressions, hierarchic urination, false pregnancies and destructive behaviour may appear between others (Pageat, 2000). Aggressive behaviour may be directed to one or more members of the household, depending on the relation with the dog, their relative status and their ability to control the dog (Mertens, 2006)

3.3.2.1.2 Behaviour modification treatment

Prior to anything else, measures involving the environment must be taken, such as physical barriers to avoid injuring human beings and also other animals, and teaching the dog how to wear a muzzle. Punishment and confrontation must be avoided at all costs. Therapy will begin by ignoring the dog completely during a period of time, afterwards to be ignored systematically, which implies not answering to any request of interaction made by the dog as it would increase its control.

The owner is to begin any sort of interaction, ignoring the dog's initiative to establish any sort of communication. The dog's access to his valued resources is to be controlled, and only if the dog obeys an order, for example "sit", it will be rewarded with the resource. It should also be avoided that the dog repeats aggressive manifestations, detecting all of the situations in which they usually appear. The reinforcement of basic education should be done to acquire a greater control over the dog in all of the situations. It is recommended to practice obedience exercises with the leash and muzzle if needed for ten minutes on a daily basis. As behaviour modification techniques, desensitization and counterconditioning are recommended.

The treatment's protocol is based on reducing the stimulus that induces the aggression to the point where the dog doesn't react. The time exposed to the stimulus is increased as it becomes better tolerated, avoiding at all costs an aggressive response, but if so, the treatment is to be restarted from the beginning (Overall, 1997; Mertens, 2006).

3.3.2.2 Intraspecific aggressiveness

This sort of aggressiveness happens between individuals from the same species. Dogs may be aggressive towards other dogs the same way they are aggressive towards people as we have previously mentioned. There are two different forms, depending to whom the aggression is directed: towards unknown dogs and to the ones with which they live, these are known as "fraternal rivalry" and "sociopathy" (Pageat, 2000). Fights between unknown dogs are more common; however, they're less harmful than those between known dogs and predominantly imply males that do not know each other (Mertens, 2006).

When conflictive situations appear in dogs that live in groups (2 or more) the most classic symptomatology is the increase in frequency of the hierarchic aggressive behaviours. This happens because of an alteration in the hierarchic organization, in which the owner's

presence is a factor that provokes the aggression, since the tendency is to interfere in said situation, which produces a worsening and perpetuation of the conflict, because it stops the conflict from developing naturally until one of the protagonists submits. The information about the stimuli that provoke the fights is fundamental to establish how to act in these situations. This sort of aggression tends to happen in the situations that include competing over valued resources and whose goal is to establish a dominance- subordination relation (Mertens, 2006).

3.3.2.2.1 Diagnosis

Dogs that attack unknown dogs may have different motivations. The diagnosis should be accompanied by defining the subjacent factors such as fear, territoriality, competition or a learnt behaviour consequence of a specific training or an unintentional or unnoticed reinforcement. These factors may occur in different combinations and aren't exclusive between themselves. To determine the dog's motivation, everything related to corporal postures, the victim's characteristics, place of fighting, situations that cause the fights and the responses made by the owner in these situations should be observed (Overall, 1997).

Diagnosing aggressiveness between dogs that know each other is more common in same sex dogs and it implies the existence of one of the two following elements: alteration of the behaviour that appears after introducing a new individual to the pack, or alterations that appear after the beginning of the sexual maturity of one of the pack's members. These elements are associated to the impossibility of the animal's ability to carry out the combat until the point of submission of one of the adversaries or the impossibility for the defeated to stay away from the group.

For the diagnosis it is also interesting to observe if there is an increase in the aggression's frequency, urination and hierarchic mounting (Pageat, 2000). Dogs with this type of aggression suffer a high state of anxiety which doesn't allow them to understand their role in the hierarchy (Overall, 1997). The subordinate dog may avoid encounters, give up his place to the other dog and take on submissive postures when the other dog approaches. The highest rank individual tends to respond when the submissive tries to access a resource (Mertens, 2006).

3.3.2.2.2 Behaviour modification treatment

Treatment directed towards aggressiveness between strangers consists mainly on behaviour modification therapy. The owner's control over the dog must increase through obedience, doing basic education exercises daily meant to avoid and control aggression towards other dogs. Attacking dogs must be controlled with a muzzle and leash in public places to prevent injuries. Owners should work with their dogs desensitization and counterconditioning techniques to replace the unwanted behaviour with the wanted behaviour such as sitting. As soon as it obeys it must be rewarded with a treat. This exercise should begin at a distance that will allow the dog to stay calm and centre its attention towards its owner; this distance should be reduced as the dog is capable of tolerating the approach without showing any signs of aggressiveness. It is convenient to practice these exercises daily in short 10 to 20 minute sessions, once or twice a day (Overall, 1997; Mertens, 2006).

In the treatment of aggressiveness between known dogs it is fundamental to inform and the counsel the owner in the meaning of the hierarchies and the canine expectations in the group, for the compliance of the proposed rules. Therapy should be systematic and should allow the group to reorganize using its own mechanisms. About the environment: the separation of the dogs by physical barriers is indicated.

First of all the dogs' hierarchic rank should be established and once it is set which is the highest ranked dog, you should insist on conveniently organizing the space, allowing the submissive dogs to stay away from the high ranked dog's sight. The subordinate dog should be ignored, at least for a time, and receive the owner's attention, but not at the expense of the dominant dog's attention time. The dominant one, will go through the doors first, will eat first, receive exclusive attention from its owner and will have access to its favourite resting spots (Mertens, 2006). Lastly, castration has also been a recommended technique which is relatively successful, but it should be applied over the subordinate and only if its inter-male aggressiveness.

3.3.2.3 Territorial aggressiveness

Territorial aggressiveness tries to stop intrusion in the territory (Pageat, 2000). According to Mertens (2006) territorial aggressiveness is mainly protective and, because of this, can be based on fear. A fear response is triggered by a perceived threat towards a valued resource. The majority of individual approaches towards the territory will pass and disappear; however, the fact that an individual disappears may serve as a powerful reinforcement of the aggression. It presents itself when the aggressive behaviour is directed towards a person or animal that is not considered part of the pack. Aggression may be directed towards people or animals that approach a member of the family or the property perceived by the pet. The term perceived property is used because there is no guarantee that the dog may know the limits of the conventional property (Landsberg, 2003).

3.3.2.3.1 Diagnosis

The key aspects of the diagnosis are that this sort of aggressiveness only shows itself towards strangers and only when they enter what the dog considers to be its territories (Manteca, 2003). Signs are the typical aggressive attitudes (upright ears, tail held high with constant wagging, an assertive posture with the weight directed forward, onslaught and biting) and vocalization (growling, barking, etc). This behaviour can be observed in males, as well as females and it generally appears for the first time before the age of 3 (Landsberg, 2003).

3.3.2.3.2 Behaviour modification treatment

The territorial aggressiveness behaviour modification treatment should be mainly directed to avoid damages towards people and other animals through physical barriers. It is equally recommended to isolate the dog while there are guests and train him in the use of a muzzle. The reinforcement of obedience through daily basic education exercises is aimed to achieve the owner's control over the dog. The use of desensitization and counterconditioning with the progressive approach of people, under the use of a leash is indicated. Move the exercise towards the entrance, presenting triggering stimuli such as ringing the door bell, letting guests and others in, reinforcing through treats, the wanted behaviours.

3.3.2.4 Fear aggressiveness

Aggressiveness because of fear is one of the ways in which the dog expresses its anxiety towards certain stimuli. An organism that finds itself in a situation from which it is incapable of withdrawing from will respond aggressively. It will attack its adversary without going through the phase of intimidation and without controlling the intensity of the aggression. This aggression tends to be accompanied by neurovegetative manifestations (Pageat, 2000). It is probably the second most frequent form aggression that lacks an organic cause and is directed towards people, after dominance aggressiveness. It happens in the same frequency both in

males and females. The efficient elimination or withdrawal of the aversive stimulus reinforces this behaviour. Insufficient socialization (lack of contact with people during the sensible period, between 3 and 12 weeks of age) and inconvenient punishment or traumatic experiences are frequent causes of fear aggressiveness (Landsberg, 2003; Manteca, 2003).

3.3.2.4.1 Diagnosis

The key element in diagnosing fear aggressiveness is the dog's posture. At first it shows a distinctive defence aggressiveness posture, with its tail tucked between its back legs, lowered crupper, ears directed backwards and, in occasions piloerection. It also tries to not approach the person, avoiding contact and only being aggressive as a last resource. It is important to take in consideration that it isn't always like this, since sometimes it behaves as if it wanted to lunge itself at the person. However, a detailed observation of the animal gives clues that the subjacent motivation is fear, some authors sustain that barking frequently accompanies the signs of this behaviour (Manteca, 2003).

3.3.2.4.2 Behaviour modification treatment

The treatment for fear aggressiveness is made of a behaviour modification program that consists of desensitization and counterconditioning. To begin with it is important to consider that dogs should be restrained with leashes and muzzles to avoid damage to humans during their training. Obedience should be reinforced through basic education exercises to obtain the owner's control over the dog. Foresee a whole situations that provoke a fearful response and avoid them. During desensitization and counterconditioning, exposing the animal to stimuli that cause fear should be progressive and controlled, and with the stimulus at a distance.

3.3.3 Pharmacological treatments for aggressiveness disorders

Prescribing psychotropic drugs (see anxiolytics table, dosage and indications) may be necessary to allow the dog to learn the necessary techniques to correct each type of problem. Psychotropic drugs tend to place the aggressive dog with high anxiety levels, in a more emotionally equilibrated state. Like selective inhibitors of the serotonin reuptake, tricyclic antidepressants have anxiolytic, anticonvulsants and antiaggresive effects (Crowel-Davis and Murray, 2006).

Anxiolytics may be useful to control aggressiveness in dogs with history of anxious behaviour. To obtain the expected effects at treatment 6 to 8 weeks long may be necessary, while the behaviour modification techniques are applied. In some cases a long term use is necessary, which will vary in accordance to the achieved control over the dog's anxiety. Fluoxetine's effect on reducing dominance aggression has been confirmed after three weeks of administration (Dodman et al., 1996). Equally, in short treatment periods of 10 weeks, it has been confirmed that the use of Psychotropic drugs results in good results in aggressiveness disorders related to dog anxiety, (Ibáñez and Anzola, 2009). This is why the use of Psychotropic drugs is an efficient tool in the therapy of aggressive dogs (see table of anxyolitics, dosage and indications). Psychotropic drugs may have unpredicted effects, including the increase of agitation, which will lead to an increase in the clinical risk (Reisner, 1999), this is why they must be use with precaution.

3.4 Compulsive disorders

Dogs suffer from repetitive behaviour disorders which are not well defined. Different authors have used different denominations such as "stereotypes", "obsessive compulsive disorder" or "compulsive disorder". It is a nosological entity linked to anxiety and defined

by Pageat (2000) as "the presence of obsessions or compulsions serious enough to be responsible of a clear ill-being or a functional handicap".
Compulsive disorders in animals are related to states of anxiety and it is frequent to find these sorts of reactions in dogs that suffer stress. When the stress factor is chronic or unpredictable, the animals tend to show inappropriate or excessive behaviour responses, in order to reduce the level of excitement and consequently the harmful effect of the prolonged physiological response (Dantzer and Mormede, 1985). If the abnormal behaviours are repeated, they can become learn responses when proven efficient in reducing the emotional negative response and the stress associated response. Substitutive behaviours that redirect the energy towards another activity may appear, called displacement activity, which manifests itself in a repetitive and stereotyped way such as licking or "grooming" (Mason, 1991).
Currently there is a dilemma whether dogs really experience the equivalent to human's obsessive compulsive disorder or just a compulsive disorder; in this chapter we use the term obsessive compulsive disorder just like other authors (Beaver, 1999; Overall, 2007; Pageat, 2000) since we consider that animals can have obsessions, although doubtlessly different those of human beings. The obsessive-compulsive disorder is a recognized disorder by animal psychiatry and one of the most disabling (Dell 'Osso et al., 2007). Luescher (1998) calls them compulsive disorders and indicates that they are abnormal behaviours produced in repetitive and invariable ways, and that interfere with normal behaviour. Compulsion is defined as a repetitive and intentional behaviour manifested in a stereotyped way and that presents itself as the response to an obsession; compulsive behaviour is not pleasant, it is executed with the aim to reduce the state of anxiety (Hollander, 1993).
According to Diagnosis and Mental Disorders Statistics guide (DSM-IV) for humans, the obsessive compulsive disorder (OCD) is defined as the presence of obsessions and recurrent compulsions that cause a strong stress or functional alterations (Jang et al., 2010) and which are included in the group of anxiety disorders by the American Psychiatric Association (2002). The behavior syndrome in animals might not be wholly analogous to obsessive-compulsive disorder in humans, although they might share a similar pathophysiology.

3.4.1 Diagnosis

An OCD diagnosis requires that the symptoms provoke a notorious ill-being, with a time determined duration and that it significantly interferes in the animal's normal functioning (Eissen et al., 2009). The course of this disorder is chronic and increases and diminishes in severity, frequently as a reaction to stress. Normally, in humans other psychiatric disorders coexist such as mood disorders, anxiety and psychotic disorders (Dell'Osso et al., 2007). We have been able to observe that in dogs there also exists a relation with mood and anxiety. The proportion in the animal population is high due to genetic causes, mainly because of the procedures of genetic selection, which include inbreeding as a common practice (Robins et al., 1984).
In animals, obsessive-compulsive disorders have been divided in three categories: conflict behaviors, emptiness and stereotypes. Conflict behaviors have been associated to restriction and impoverishment conditions, for example cannibalism, urine suction and tics (Wiepkema, 1980). Empty behaviors or empty activities are unconscious actions practiced in the absence of the stimulus under which they would be expressed and that do not pursue any purpose; in dogs empty behaviors such as licking, self-mutilation and masturbation are typical (Landsberg et al., 2003). The diagnostic signals of stereotyped behaviors in dog may vary a lot, and some may be more frequent than others, such as licking the nose and lips,

shaking the head, yawning, circling, pacing, tail-chasing, self-mutilation, snapping at the air, excessive grooming, and rhythmic barking (Landsberg et al., 2003; Pageat, 2000).
Stereotypes may have their origin in an organic disorder, thus a neurological exam and a blood analysis are necessary; and in some determined cases a complete dermatological protocol may also be useful. If the problem began when an important change in the environment took place or it appears under determined circumstances, it is probably a stereotype with no organic cause.

3.4.2 Treatment

Like in the majority of behaviour disorder cases, the treatment requires the intervention over the animal's environment, the behaviour modification and the administration of psychotropic drugs. For these disorders treatments combining the use of psychotropic drugs and behaviour modification techniques have been effective, especially habituation and counterconditioning (Overall, 1997). The treatment mandatorily associates chemotherapy and a behaviour therapy (Pageat, 2000).
The reduction of anxiety, or the discovery of methods used to reduce the source of excitement and conflict are the first aspects of the treatment to be investigated in compulsive behaviours. Another important aspect to value in the treatment is the incompatibility in the dog-owner relationship, which may cause problems and in a very special way when it involves an incoherent education. Stereotypes have a reserved forecast and the recommended treatment isn't always successful.

3.4.2.1 Intervening in the environment

The environment in which the animal lives must be studied to make sure that the dog has the resources that will offer enough stimulation. Time used for playtime, exercise and attention as well as how they are administered should be analyzed. It is very important to identify and eliminate, if possible, the environmental factors responsible of the compulsive behaviour.
It has been proven that environmental enrichment systems are very efficient, mainly those related to food and social aspects through games with toys or other living beings, if the environment is very poor in stimuli. It is the case of those animals that are locked up for long periods of time on their own in places of small dimensions or with very few occasions of social interaction. In many cases, triggering game sequences where contact with the owner is encouraged resulting in good results which is why it is recommended to practice sports activities where the activity is shared and a correct communication is established between the dog and owner such as that which is done during the practice of "agility". In other occasions, the best treatment is to take the animal to live in a different environment.

3.4.2.2 Behaviour modification techniques

An obedience training program that completes the possible found deficits should be started (Landsberg, 2003). If anamnesis indicated a fear problem or it is associated to an anxiety separation problem, the treatment must include a protocol such as those suggested in each of the cases previously described.

3.4.2.3 Pharmacological treatment

The serotonin reuptake inhibitors (ISRS) and the cognitive-behavioural therapy represent the first line of treatment for OCDs and related disorders (Dell 'Osso et al., 2007). It has been found that clomipramine is effective and well tolerated in the therapy of obsessive compulsive disorders (Seksel and Lindeman, 2001), likewise fluoxetine has resulted efficient as a

complementary tool in the therapy of this disorder (Ibañez and Anzola, 2009). The drugs that result to be the most useful are clomipramine, fluoxetine and selegiline (Manteca, 2003). It is important to take in account that none of them reduce or eliminate stereotypes in all of the treated cases, which is why sometimes it is necessary to try various treatments before obtaining a satisfactory result. See table of anxyolitics, dosage and indications for dogs.

4. General therapeutic management of anxiety in dogs

As we have described in the most important anxiety related disorders, the therapeutic management is the implementation of a battery of combined therapies to reduce the state of anxiety, which will depend on the degree of intensity and if there is an involvement brain neurochemistry. The best tools to correct or minimize behaviour disorders consequence of anxiety are behaviour modification techniques and the use of psychotropic drugs. Therapy must begin with a relaxation protocol which predisposes the animal to a better acceptance of the upcoming treatment. Afterwards, psychopharmacology is used and finally behaviour modification techniques such as habituation, desensitization and counterconditioning.

4.1 Relaxation

The first step is to establish a model of tranquil and relaxed responses from the dog when the provocative stimulus isn't present. It is very important that owners recognize their pet's tranquil responses and that they reward them accordingly. It is advisable to include within therapy at least two daily sessions of relaxing massages which must be set in a tranquil place, without any noise and if possible with relaxing background music. It is convenient to do these sessions during 10-20 minutes until making the animal reach a relaxed state in which it almost falls asleep. During the sessions the animal should remain seated or lying down and be rewarded for its tranquil and relaxed attitude. The owner may gradually add some distractions during these exercises, such as clapping or moving a few steps away from the dog and then returning to the initial position (Neilson, 2006).

4.2 Pharmacological treatment

Alterations in dog's behaviour are problems that majorly correspond to neurochemical imbalances triggered by high levels of anxiety. It is more and more frequent in veterinary medicine that owners are interested in finding help for their animals which have anxiety related disorders. Psychotropic drugs are used to compensate the imbalance in the chemical substances of the central nervous system. Since long ago, anxiolytic drugs have been used in humans and animals.

In general, psychotropic drugs result in modifying the animal's response capacity to successfully confront external stimuli, helping its homeostasis to improve, which will reduce the levels of anxiety. Anxiety blocks the learning mechanisms, incapacitating the animal to respond efficiently and making it even more susceptible to anxiety, generating a vicious circle. Some behaviour disorders may ne reduced both in frequency as well as severity with the use of psychotropic drugs which produce modifications in the neurotransmitters (Mills and Simpson, 2006).

Tricyclic antidepressants, benzodiazepines and other drugs with anxyolitic properties induce a control over many dog's anxiety (King et al., 2000). Serotonin reuptake selective inhibitors (ISRS) are classified as antidepressants and their use in veterinary is due to its anxyolitic effects. Fluoxetine is the most used in pets to treat behaviour problems and its most frequent use includes the treatment of anxiety (Crowell-David and Murray, 2006).

Benzodiazepines are part of the most used pharmacological group in the treatment of anxiety nowadays. They are synthetic compounds that strengthen GABA's effects (Cuenca and Álamo, 2005). They are also an alternative to supplement the fluoxetine's effect in the treatment of anxiety (Ibáñez and Anzola, 2009).

4.3 Behaviour modification techniques

The main behaviour modification techniques used in veterinary are habituation, systematic desensitization and counterconditioning. Habituation and counterconditioning are use to increase the threshold in which the animal responds to a specific stimulus or situation. This procedure requires the identification and ranking of the aversive stimuli, the animal relaxation training, the identification of the animals responses at an acceptable level, the presentation of the stimuli that trigger the problem, by order of range, and the reinforcement of learning (Mills, 2006).

Counterconditioning leads to the extinction or the control of the unwanted behaviour; this is achieved by teaching the dog another behaviour, in this case wanted, which interferes competitively with the execution of the unwanted behaviour. Once the dog learns how to do the competitive behaviour which is incompatible with the unwanted behaviour, a desensitization technique can be started (Overall, 1997).

5. Clinical trials

In the Animal Behaviour Clinic of the Veterinary Faculty, of the Complutense University of Madrid, we have carried out a series of several clinical trials studying a therapeutic efficacy of different drugs in anxiety states in dogs. Psychotropic drugs i.e. antidepressants and anxiolytic and behaviour modification techniques have been used. The efficacy of the used methods in the different dosage regimes has been studied. All anxiety disorders in dogs have been grouped into two general categories in accordance with clinical casuistic: the disorders with and without anxiety and aggressive behaviour.

We have experienced several benzodiazepine drugs together with the fixed therapy. In addition, fluoxetine and a common behaviour modification technique has been also used. The main published results concerned the use of diazepam, while the data on the clorazepate and alprazolam administration have not been published yet. There exists possibility in efficacy discrepancies and adverse events between different benzodiazepines. The possible differences in treatment efficacy in relation to gender and age of the studied animals' also exist.

In the first study 40 dogs from different breeds, age and gender with anxiety disorders were included (Journal of Veterinary Behaviour, 2009, 4, 223-229). Fluoxetine, diazepam and behaviour modification as therapy methods have been used. The dogs were grouped into two diagnostic categories, according to presence or absence of anxiety and aggressive behaviour. The dogs were also classified in 4 other groups: castrated, whole, young and adult. Diazepam was used orally at a dosage of 0.3 mg/kg once a day for 4 weeks; fluoxetine was given orally and daily at a dosage of 1mg/kg during 10 weeks. Likewise, a behavior therapy was started from the first day of the treatment.

Obtained results have shown a great improvement or elimination of the clinical signs in 38% of the dogs, a minimal or moderate improvement in 31%, while 24% of the dogs didn't achieve any improvement. There was no evidence of difference in the treatment efficacy between the anxious and aggressive groups; castrated and whole; and young and adult. In addition, a positive correlation between the owner's compliance with the therapy and an improvement results was revealed.

Drug	Dosage (PO)	Indications
Amitriptyline	1,0-6,0 mg/kg q12h	Compulsive disorders, aggressiveness and separation anxiety
Alprazolam	0,02-0,1 mg/kg q4h-12h	Generalized anxiety, fears and phobia, aggressiveness and separation anxiety
Acepromazine	0,5-2.0 mg/kg q8h-12h	Noise phobias
Buspirone	0,5-2.0 mg/kg q8-12h	Fears, separation anxiety
Clomipramine	1,0-3,0 mg/kg q24h	Generalized anxiety, fears and phobias, separation anxiety, compulsive disorders and aggressiveness
Clonazepam	0.1-0.5 mg/kg q8h-12h	Aggressiveness, Generalized anxiety
Chlordiazepóxide	0.2-1.0 mg/kg q12h	Generalized anxiety
Diazepam	0,5-2,0 mg/kg q4h	Generalized anxiety, fears and phobias, aggressiveness, capricious alimentary behaviour and separation anxiety
Clorazepate	0,5-2,0 mg/kg q4h	Generalised anxiety and separation anxiety
Doxepin	3.0-5.0 mg/kg q8h-12h	Compulsive disorders, fears and phobias
Fluoxetine	1.0-2.0 mg/kg q24h	Separation anxiety
Haloperidol	0,05-2.0 mg/kg q12h	Compulsive disorders and aggressiveness
Imipramine	0,5-2,0 mg/kg q8h	Urination because of submission and excitement
Lorazepam	0.02-0.5 mg/kg q8h-24h	Generalized anxiety
Oxazepam	0.2-1.0 mg/kg q6h-24h	Generalized anxiety, anorexia
Paroxetine	0,5-2.0 mg/kg q24h	Generalized anxiety
Selegiline	0,5-1,0 mg/kg q24h	Aggressiveness, cognitive dysfunction and compulsive disorders
Thioridazine	1.0-3.0 mg/kg q12h-24h	Generalized anxiety, aggressiveness, compulsive disorders, fears and phobias

Charney et al., 2006; Crowell-Davis & Murray, 2006; Landsberg et al., 2003; Mills & Simpsom, 2006; Overall (1997); and Simpson et al., 2007.

Table 1. Main drugs, dosage and indications for dogs.

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15

MicroRNA-Mediated Regulation and the Genetic Susceptibility to Anxiety Disorders

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1. Introduction

Investigation of how genetic variation within complex gene regulatory networks results in phenotypic alterations may represent a useful approach towards the understanding of human evolution and disease. In this regard, genetic studies can contribute to the identification of genes and pathways underlying the susceptibility to psychiatric disorders including anxiety disorders. However, this has shown to be difficult due to the complexity of both, the genetics and the phenotypes of these disorders. In fact, even though the estimated heritability of psychiatric disorders is high, most genetic risk alleles for these disorders have still not been identified, leading to the conclusion that either major risk alleles are scarce or that they increase risks only marginally, that is to say that their associated Odds Ratio is low. Genetic heterogeneity for complex disorders is widely accepted and, in addition, it has been suggested that non-standard factors, such as epigenetic or regulatory changes or combinations of various of these elements could be involved in the aetiology of psychiatric disorders (Burmeister et al., 2008). Accordingly, increasing evidence at a population and experimental level indicates that genetic variation at regulatory regions underlies differences in gene expression and could be a major contributor to phenotypic diversity in human populations (Buckland et al., 2005; Knight, 2005; Rockman and Wray, 2002). This may be particularly true in the case of psychiatric disorders, where changes in regulatory elements leading to small variations in the dosage of proteins involved in neuronal pathways may disrupt the fine-tuned equilibrium of complex brain functions and contribute to the development of the disease. In this respect, even though the search for susceptibility genes for anxiety disorders has led to the finding of positive associations, most of these studies have produced results that are inconsistent or not clearly replicated, indicating that the genetic basis of anxiety disorders requires further investigation using alternative approaches.

Stress has also been shown to have a critical role in the development of anxiety disorders (Lupien et al, 2009), at least partially, through mechanisms related to neural plasticity. Synaptic connections in the brain undergo experience-dependent functional or morphological changes through complex pathways that are not yet fully understood, but for which microRNAs (miRNAs) might have a critical role (Kosik et al., 2006). miRNAs are endogenous small non-coding RNAs that regulate gene expression by means of partial

complementarity to miRNA binding sites at their target genes. These molecules have emerged as key regulators of almost every biological process including accurate control of neuronal gene expression (Krol et al., 2010). Due to their enormous regulatory potential, miRNAs could be considered as one of the most significant discoveries in molecular biology of the last decade.

This chapter aims to give insights into the role of gene regulation and, in particular, into the involvement of miRNAs in the pathophysiology of anxiety disorders, with special focus on panic disorder. We will explore the hypothesis that changes in miRNA-mediated regulation, originated from changes in the miRNAs themselves or on their target sites, may alter the dosage of proteins involved in fundamental pathways for brain function, affecting the precise homeostasis of the central nervous system and contributing to the development of anxiety disorders. The chapter will go through recent research results that, by a combination of association analyses and functional approaches, involves particular miRNAs and several candidate genes in the susceptibility for anxiety disorders and indicates that polymorphisms affecting miRNA-mediated regulation may be determinant of a range of human traits related to anxiety (Muiños-Gimeno et al., 2009; 2011).

2. Anxiety disorders

Even though definitions, assessments and classifications of mental disorders may vary along time, guideline criteria listed in the International Statistical Classification of Diseases and Related Health Problems, Diagnostic and Statistical Manual of Mental Disorders and other manuals are widely accepted by mental health professionals. According to the latest version from the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revised (DSM-IV-TR) (American Psychiatric Association, 2000), anxiety disorders include a broad category of heterogeneous disorders where the primary feature is abnormal or inappropriate anxiety. The central and unifying features of all anxiety disorders are heightened sense of arousal or fear that is episodic or continuous and may be related to exposure to a specific trigger. There are both psychological and physiological components to anxiety disorders such as worry and fear or increased heart rate and sweating. Symptoms of anxiety are also part of a normal process called the 'fight or flight' phenomenon. This means that the body is preparing itself to either fight or protect itself or to flee a dangerous situation. These symptoms become problematic when they occur without any recognizable stimulus or when the stimulus does not warrant such a reaction. Population prevalence of these disorders is approximately 16%, when considering developed and developing countries (Kessler, 2009), and the age at onset is variable, some of them starting in the early childhood (Kessler, 2007).

Biological theories on anxiety disorders suggest abnormalities of the neurobiological pathways associated with modulating normal and pathological fear and/or stress states. In fact, animal models of stress have delineated major components of the stress response (Sullivan et al., 1999). However, nowadays, imaging studies also seek to identify the unique patterns and combinations of activated or deregulated brain regions involved in certain anxiety disorders (Gorman et al., 2000). A model that focuses on the amygdala and its interconnected structures has been proposed for anxiety disorders. The amygdala and hippocampus are important nuclei of the limbic system that regulate emotions and memory

storage, respectively. The amygdala seems to play an essential role in the acquisition of conditioned fear and the expression of innate and learned fear responses (Li et al., 1996). Specifically, the model describes 2 parallel pathways carrying information to the amygdala: the thalamic-amygdala pathway and the cortico-amygdala pathway. Sensory information (coming from either one out of these two pathways) enters the lateral amygdala, from which processed information passes to the central nucleus. The central nucleus, then, acts as the central component of the fear neural circuitry and projects to multiple brain systems involved in the physiologic and behavioural responses to fear. Projections from the amygdala to the hypothalamus take 2 primary routes: 1) the lateral hypothalamus, leading to sympathetic activation; and 2) the paraventricular nucleus, leading to activation of the hypothalamus-pituitary-adrenal axis.

Nowadays the DSM-IV-TR classifies anxiety disorders into several disorders: acute stress disorder, agoraphobia (with or without a history of panic disorder), anxiety disorder due to general medical condition, generalized anxiety disorder, obsessive-compulsive disorder, panic disorder (with or without agoraphobia), phobias (including social phobia), posttraumatic stress disorder and substance-induced anxiety disorder. However, categorical classification of these disorders has not been static during the years and remains still controversial (American Psychiatric Association, 2000). Problems arise from the overlapping of phenotypes within the broader context of anxiety disorders, variable expressivity of panic and anxiety or depression, and the presence of phenocopies within a family. On the other hand, since the diagnostic remains purely clinical, genetic studies are arduous to perform. The use of dimensional personality traits, such as shyness, behavioural inhibition and neuroticism, in order to better define an anxiety phenotype instead of clinical diagnosis has been proposed as a solution to this problem (Hettema et al., 2001; Kessler et al., 2005; Smoller and Tsuang, 1998). Within anxiety disorders, in this chapter we will mainly focus on panic disorder and obsessive-compulsive disorder

2.1 Panic disorder

The DSM-IV-TR defines panic disorder as the spontaneous, unexpected occurrence of panic attacks followed by persistent concern, worry, and anxiety about having additional panic attacks. Panic attacks are defined as a discrete period of intense fear or discomfort (no more than 30 minutes) that develops abruptly and reaches a peak within 10 minutes, in which at least 4 of 13 symptom criteria are met. Some of these criteria include cardiac palpitations, sweating, feelings of choking, fear of losing control, and fear of dying. Panic attacks often mimic symptoms of physical complaints such as a heart attack or other life-threatening illnesses (American Psychiatric Association, 2000) and typically occur spontaneously, with no apparent trigger. However, there is evidence that shows that for the majority of patients mild phobic or hypocondriacally symptoms precede the panic attacks. It has been therefore proposed that panic attacks are more unpredictable than unexpected (Gratacos et al., 2007). Panic disorder may manifest with or without accompanying agoraphobia. However, agoraphobia can also occur without panic disorder, and panic attacks can occur in the absence of panic disorder. Comorbidity with depressive and addictive disorders is frequent as much as a lifetime prevalence rate of 50-60%. Life prevalence of panic disorder is 1- 2% (Regier et al., 1990) and twice as many women suffer from the disorder if compared to men (Weiller et al., 1998; Weissman et al., 1997). Panic disorder has a bimodal age at onset

distribution, with highest incidence in late adolescence and a second peak in the mid 30s (Sansone et al., 1998). This disorder is, however, less often observed in the elderly (Gratacos et al., 2007). Panic disorder has moderate estimated heritability rate of 44% (van den Heuvel et al., 2000), and results from a meta-analysis performed on genetic epidemiology studies showed that there is a significant association between panic disorder in the probands and panic disorder in the first-degree relatives ($p<0.0001$) (Hettema et al., 2001). Furthermore, risk for panic disorder increases in adult first-degree relatives, when the age at onset of the proband is under twenty years of age (17-fold to 6-fold), suggesting that age at onset might be useful in differentiating familial subtypes of panic disorder (Goldstein et al., 1997). In addition, panic disorder and agoraphobia with panic attacks were shown to be more than five times more frequent in monozygotic twins than in dizygotic twins (Torgersen, 1983). This was further corroborated by a later study which found a significantly higher concordance among monozygotic than dizygotic twins for panic disorder (73% vs. 0%), confirming a role for genetic factors in panic disorder, but not for spontaneous panic attacks (57% vs. 43%) (Perna et al., 1997). Agoraphobia is also thought to be a more severe variant of panic disorder as suggested by the fact that the risk of panic disorder is increased among the relatives of patients with agoraphobia (8.3%) and the relatives of patients with panic disorder (17.3%). However, while the risk for agoraphobia is also increased among the relatives of patients with agoraphobia (11.6%), it is not among the relatives of patients with panic disorder (1.9%) (Noyes et al., 1986).

2.2 Obsessive-compulsive disorder

Obsessive-compulsive disorder is characterised by obsessions and compulsions. Obsessions are recurrent intrusive and unwanted thoughts that the sufferer cannot dispel. Common themes of the obsessive thoughts include thoughts that the person may cause harm to others or that harm may befall others, or thoughts that the person or others are contaminated. Other common themes are centred on the need for order, symmetry or perfection. The obsessive thoughts are associated with negative feelings, usually anxiety, but other emotions such as disgust, guilt or shame may also be experienced. As a response to these feelings generated by the obsessive thoughts, the person may perform compulsions, and performance of the compulsions temporarily decreases the negative effect. The compulsions are stereotypic, ritualised behaviours that are usually observable but which may include covert mental rituals. Common rituals include repetitive checking, washing or cleaning, or repetitive rearranging and ordering of objects. Examples of covert mental rituals include repetitive counting, praying or thinking magical statements (Gelder et al., 2001). In summary, the obsessions and compulsions are distressing, time-consuming, and often lead to impairment in occupational, scholastic, or social functioning. According to the DSM-IV-TR to meet criteria for obsessive-compulsive disorder, the individual must report ineffective efforts to resist, neutralise, or suppress thoughts or behaviours with other thoughts or actions. In addition, the thoughts must be distinct from those associated with other anxiety disorders, and the individual must acknowledge that the thoughts are a product of his or her own mind (American Psychiatric Association, 2000). The disorder affects approximately 1–3% of adults (Kessler et al., 2005) and is ranked by the World Health Organization as among the ten most disabling medical conditions (Grisham et al., 2008). Although there is strong evidence that obsessive-compulsive disorder has a genetic component (Hettema et

al., 2001), definitive single domain or integrative models have not yet been established (Stein, 2002). The broader obsessive-compulsive disorder phenotype has been divided into subgroups that are potentially more etiologically and genetically homogenous: symmetry/order, aggressive/checking, contamination/cleaning and hoarding (Mataix-Cols et al., 2005). A more controversial fifth dimension may be also included, consisting of somatic, sexual, religious obsessions and mental rituals (''pure obsessional'') (Mataix-Cols et al., 2006). Obsessive-compulsive disorder usually begins before 25 years of age and often in childhood or adolescence.

3. Problems of the genetic study of anxiety disorders - high level of regulatory control in brain

The hereditary basis for psychiatric disorders was already recognised at the turn of the nineteenth century. After that, genetic influence on all major psychiatric disorders has been consistently demonstrated by twin and adoption studies (Plomin et al., 1994). In fact, estimated heritabilities for bipolar disorder, schizophrenia and autism -80% to more than 90%- (Bespalova and Buxbaum, 2003; Gupta and State, 2007; Kieseppa et al., 2004; McGuffin et al., 2003; Sullivan et al., 2003) have been shown to be much higher than that of breast cancer -5% to 60%- for instance (Locatelli et al., 2004; Schildkraut et al., 1989), for which several genetic factors are now well established (Plomin, et al., 1994). However, although genetic influences on psychiatric disorders have been well established, localization of genes responsible for these effects has proven to be extremely difficult. This is probably due to several reasons, among others: Problems arising from the difficult diagnosis of Psychiatric disorders, the probable multifactorial and polygenic origin of these disorders and the high level of regulatory control and gene interactions to what human brain and behaviour are exposed.

Regarding the latter, the search for susceptibility genes for anxiety disorders has classically focused on neurotransmitters and members of neurotransmitter synthesis and degradation pathways, although other groups of molecules including genes involved in neurodevelopment and synaptic plasticity have been also studied more recently. Nevertheless, the complexity of the central nervous system requires not only of a precise function of its formal components but also of an accurate gene regulation of the system. Hence, genetic variation in regulatory regions is nowadays recognized as a major contributor to phenotypic diversity (Buckland et al., 2005; Knight, 2005; Rockman and Wray, 2002). Taking into account the importance that minimal changes in gene regulation could have in gene dosage, and gene dosage in turn, to genetic susceptibility to disease, regulator elements acting in the brain should be included in every psychiatric candidate gene study. Accordingly, post-transcriptional regulation, previously underestimated, is growing in importance and is thought to play an important role on mammalian development and disease (Sun and Tsao, 2008). Contrary to what was thought during many years, it is nowadays accepted that more than half of the human genome is transcribed and that most of the generated transcripts (~98%) are actually non-protein coding (Mattick, 2009). RNAs that do not code for proteins and directly function as RNAs are called non-protein coding RNAs (ncRNAs). Importance of ncRNAs is supported by the fact that their accumulation tends to increase with organism complexity along the evolutionary scale (Mattick, 2009) and, even though a recognized function is lacking for most of these ncRNAs, they are mostly predicted to have regulatory functions (Costa, 2010) and should thus be considered as candidate genes for disease.

4. MicroRNAs

In the last few years additional species of ncRNAs have increasingly been discovered (Costa, 2010), among which, small ncRNAs, and among them microRNAs (miRNAs), attract particular attention because of their role in processes such as RNA silencing and modification (Kawaji and Hayashizaki, 2008). miRNAs are endogenous small ncRNAs that regulate gene expression by means of partial complementarity to miRNA binding sites at their target genes (Bartel, 2004). Recent estimates indicate that they regulate more than 30% of all protein-coding genes, building complex regulatory networks that control almost every cellular process (Filipowicz et al., 2008). The founding member of the miRNA family, lin-4, was identified in *C. elegans* in 1993 through a genetic screening for defects in the temporal control of post-embryonic development (Lee et al., 1993). Withal, it was not until 2001 that miRNAs were recognized as a large and phylogenetically extensive family of non-protein coding RNAs representing a new layer of gene regulation (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Since then, the number of identified miRNAs has increased vertiginously, in humans for example, their growth has ranged from none, in 2001, to a total of 1424 human miRNAs that are being recognized by the last version of the miRNA database (April 2011, Sanger miRBase, release 17.0). Remarkably, during the last 4 years (2007-2011), the number of identified miRNAs has been triplicated. Consequently, publications and knowledge on this class of small RNAs has also increased considerably. As a matter of fact, the number of pubmed publications on miRNAs was of five in 2001, while nowadays there are roughly 12000. It is estimated that miRNAs will comprise 1%-5% of animal genes (Bartel, 2004), being, in consequence, one of the most abundant classes of regulators in the genome.

On the other hand, it is known that a single miRNA can target as many as several hundred genes, and that one gene can be targeted synergistically by more than one miRNA. Taken together, miRNAs form an interconnected regulatory network that does not simply turn genes on or off, but are thought to ''tune'' the expression level of their target genes (Sun and Tsao, 2008). Post-transcriptional regulation by miRNAs may thus represent an important mechanism through which the central nervous system accomplishes its demands for precise but rapid changes in gene regulation. In fact, both the synthesis and degradation of RNAs are likely to require less time and energy than those of proteins. Consequently, non-protein coding RNAs and particularly, miRNAs, are suitable candidates for the regulation of a constantly changing microenvironment, like the central nervous system.

The importance of miRNAs is made evident by their conservation along evolution and by the multiple processes in which they are implicated, such as developmental timing, cell differentiation and morphogenesis (Stark et al., 2005), synaptic plasticity (Schratt et al., 2006), regulation of immunological functions (Pauley and Chan, 2008) and stress response (He et al., 2007). Different types of cellular stress have been shown to alter miRNA levels; for example, hypoxia-responsive transcription factors such as nuclear factor-kappa B and p53 induce miRNA genes (He et al., 2007; Taganov et al., 2006). In general, studies on oxidative stress, cold stress and nutrient deprivation indicate that long-term stress may have an impact on miRNAs and on global gene expression, perhaps leaving tissues more susceptible to pathogenic processes. It is thought that chronic stress can initiate cellular reprogramming through alterations in miRNA expression or activity, leading to sustained changes in gene expression and cellular physiology (Hudder and Novak, 2008). It is also worth mentioning that miRNAs are also involved in cell cycle progression and apoptosis (Carleton et al., 2007), as emphasized

by their implication in cancer or in endocrine regulation of energy homeostasis, (Poy et al., 2004).

In particular, miRNAs have important functions in the brain and have been involved, among others, in learning and memory (Fiore and Schratt, 2007) as well as in synaptic plasticity. The prime example of a miRNA implicated in synaptic plasticity in mammalian neurons is miR-134, which localizes to dendrites close to synapsin-positive *puncta* (Schratt et al., 2006). Its over-expression causes a significant decrease in dendritic spine size, while its depletion leads to a small increase in spine volume. miR-134 targets *LIMK1* (Lim-domain-containing protein kinase 1), whose activity is controlled by BDNF and which is involved in actin filament dynamics, a key step in the cytoskeletal modifications of spines associated with plasticity. The concomitant over-expression of miR-134 with a mutated form of *LIMK1,* that has a defective target site, rescues the alterations in spine morphology, indicating that the spine size defects caused by miR-134 are indeed due to the deregulation of *LIMK1* (Schratt et al., 2006). Furthermore, miR-134-mediated repression of *LIMK1* is relieved upon BDNF stimulation of synaptic plasticity, showing that neuronal activation intervenes to put a brake on miRNA-mediated silencing. Finally, another indication of the involvement of microRNAs in controlling local protein translation and synaptic function comes from a recent study that demonstrated that miR-128 is deregulated in HIV-1 encephalopathy (a manifestation of HIV-1 infection that often results in neuronal damage and dysfunction) and that, in addition, miR-128 inhibits the expression of SNAP25, a pre-synaptic protein that regulates Ca^{++} responsiveness (Eletto et al., 2008). The degree of complexity of miRNA pathway regulation has been revealed to be particularly high when studying neurons where several mechanisms of control have been discovered, such as the transport of miRNAs to distal sites in dendrites, the association of miRNA regulation with synaptic activation, or the reported role for the rapid miRNA turnover in neurons regarding miRNA activity-regulation (Reviewed in Krol et al., 2010).

Another interesting observation that illustrates how much more complicated miRNA-mediated regulation of mRNAs can be is based on initial studies that show that miRNAs are prone to tissue-specific RNA editing. Editing is a post-transcriptional mechanism, by which some RNA molecules are altered to contain bases not encoded in the genome (specific nucleotides are either deleted, inserted or modified to change one nucleotide into another). Such editing events alter the properties of miRNAs and seem to regulate alternative mRNA: miRNA interactions. This has been, at least, demonstrated by miR-376 targeting of a different set of genes after RNA editing in different tissues (Erson and Petty, 2008; Kawahara et al., 2007).

5. MicroRNAs and anxiety disorder

In addition to the modulation of physiological functions and due to their important regulatory role in processes of physiopathological relevance, a close relationship between miRNAs and human disease has been described. Regulatory changes that alter miRNA activity can be caused by both in *cis* and *trans* factors (relative to the locus coding for the miRNA), making possible to schematically group known miRNA-related diseases based on the specific process altered. *Cis* deregulation of miRNAs can be caused by chromosomal alterations, epigenetic modifications, polymorphic promoter elements and polymorphisms within the miRNA itself (pri-, pre- and mature miRNA sequences). *Trans* factors affecting

miRNA activity, on the other hand, include functional mutations in the proteins involved in miRNA transcription, processing and targeting, and polymorphisms in miRNA target sites (poly-miRTS). We, ourselves, based our research on anxiety disorder on the hypothesis that changes in miRNA-mediated regulation, originated from changes in the miRNAs themselves (*cis*) or on their target sites (*trans*), could alter the dosage of proteins involved in fundamental pathways of brain function, affecting the fine-tuned central nervous system homeostasis and contributing to the development of anxiety disorders. In an attempt of identifying genetic factors related to anxiety, we divided our strategy into the study of genetic variation in regulatory regions of candidate genes for anxiety disorders and the genetic study of miRNA genes *"per se"*. As described later, even though major risk alleles have not been found, results on these studies indicate that genetic variation affecting miRNA-mediated regulation may be underlying a range of anxiety related phenotypes, being the first time that miRNAs are involved in the pathophysiology of human anxiety disorders (Muiños-Gimeno et al., 2009; 2011). Table 1 shows a list of all miRNAs that have been involved in anxiety disorders.

5.1 Cis-deregulation involving miRNAs and disease

Cis-deregulation of miRNAs has been involved in several human disorders. Early clues linking chromosomal alterations in miRNA loci and disease came from observations in chronic lymphocytic leukaemia (CLL), where chromosome band 13q14, commonly lost or altered in CLL patients, was found to harbour miR-15 and miR-16 (Calin et al., 2002). Both miR-15 and miR-16 were later shown to target the 3'UTR of *BCL2*, a well- known anti-apoptotic oncogene (Cimmino et al., 2005). Initial observations that miRNA genes were located on genomic instability and fragile sites by Calin *et al.* (Calin et al., 2004) led to further analyses that have demonstrated deregulated miRNA expression profiles in various diseases.

With respect to disorders of the central nervous system an increase in miR-9, miR- 125b and miR-128 levels was detected in the hippocampus of Alzheimer's disease brains (Lukiw, 2007). Later, miRNA expression profiles of human brain tissue from individuals with Alzheimer's disease at different stages of the disease were compared (Wang et al., 2008a). This comparison reported significantly decreased miR-107 levels in patients with even the earliest stages of Alzheimer's disease. When further analyzing the role of this miRNA, the authors concluded that miR-107 might be involved in accelerated disease progression through regulation of the beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1). Moreover, other studies demonstrated that miR-29a, miR-29b-1, and miR-9 can also regulate *BACE1* expression *in vitro* and that these miRNAs were also decreased in patients with Alzheimer's disease, resulting in high BACE1 protein levels in patients (Hebert et al., 2008). On the other hand, reduced expression of miR-133b has been observed in dopaminergic neurons of Parkinson's disease patients and reduced levels of this miRNA have been thus associated with the typical degeneration of this type of neurons in Parkinson disease (Kim et al., 2007). Interestingly, distinct miRNA expression patterns have also been implicated in chronic psychiatric disorders: miR-26b, miR-30b, miR-29b, miR-195, miR-92, miR-24, miR-30e, for instance, were shown by microarray and quantitative reverse transcriptase-polymerase chain reaction to be decreased in samples from individuals with schizophrenia (Perkins et al., 2007). However, the way in which these miRNAs and their targets may be involved in common complex neurological and psychiatric disease states are yet to be examined. Concerning mutations in miRNAs or in their promotor sequences and in relation

to schizophrenia, two known SNPs, located in the adjacent +/- 100 bp genomic region of miR-206 and miR-198 were claimed to be associated with schizophrenia. One of them, in the miR-206 adjacent genomic region, remained significant after correction for multiple testing. In order to elucidate what biological signalling network might be the one affected by these miRNAs in schizophrenia, the authors performed target predictions for both miRNAs. Target predictions rendered a list of 15 genes that were predicted to be corregulated by both miRNAs. Interestingly, two of the common targets had been previously related to schizophrenia; *CCND2* had been shown to be deregulated in post-mortem schizophrenia brains and *PTPN1* had been positioned under a significant linkage peak (Hansen et al., 2007). Similar studies have implicated mutations in miRNAs or their regulatory regions with different diseases. Worth mentioning is a common SNP within pre-miR-146a that was reported to be strongly associated with papillary thyroid carcinoma. (Jazdzewski et al., 2008; 2009). This study is one of the few studies that clearly evidences that polymorphisms in miRNA coding regions can lead to disease and contrary to what is generally thought, the study proposes that mature miRNAs from the passenger strand may, as well, regulate many genetic processes. This is particularly interesting since the study uncovers the fact that miRNA processing and action are still to be deeply studied and that, as in every emerging topic of research, there might be false dogmas that should be redirected.

5.1.1 Association studies of miRNA genes with anxiety disorder

As stated in the previous section, allelic changes as well as genomic variants involving either miRNAs or their regulatory machinery may represent an important source of phenotypic variation and contribute to the susceptibility for complex disorders. Even though poorly considered until now, association studies using SNPs in miRNA genomic regions might help to evaluate the involvement of miRNAs genes in disease. In this regard, a panel of SNPs covering miRNA regions suitable for association studies was designed, constructed and used for the study of panic disorder (Muiños-Gimeno et al, 2010; 2011). Prior to the design of the SNP panel, the first step consisted in the analysis of the genomic distribution and genetic variation of all at that moment known miRNAs-containing regions. The analysis of the SNP coverage on miRNAs revealed that miRNA regions are characterized by a lower density of SNPs than the rest of the genome. In fact, at the moment of the panel design, only 24 SNPs (dbSNP 125) mapped within the miRNA sequences of 325 miRNAs, (Sanger miRBase release 7.1) -and interestingly none of them was located within a mature miRNA sequence-; this represented a density of 0.86 SNPs per kb at miRNA regions compared with the observed average of 3.99 SNPs/kb SNP for the whole human genome. The lack of mutations identified at the mature miRNAs was in agreement with the reported negative selection acting at both miRNAs as well as miRNA target sites. Existence of negative selection in conserved miRNA target sites at 3'UTRs has already been described (Chen and Rajewsky, 2006). Likewise, it has been extensively proposed that SNP density is lower in miRNA loci with respect to their flanking regions (Saunders et al., 2007). However, screening of SNPs from public databases deals with the problem of SNP ascertainment bias, mainly due to underrepresentation of low-frequency variants and the fact that not all the genome has been equally characterized. Remarkably, very recently Quach *et al*. (2009) confirmed this lower SNP density by re-sequencing 117 miRNAs in four different human reference populations, therefore avoiding ascertainment bias coming from public databases. Their analyses reported a lower SNP density in miRNAs than in other non-coding regions,

which were shown to be twice as dense. The study also showed that strong purifying selection affects the sequence corresponding to the mature miRNA (particularly the first 14 nucleotides, where no mutation is tolerated) as well as the complementary miRNA sequence (miRNA*), stem region and loop (Quach et al., 2009). In summary, these studies indicate that mutations in miRNA hairpins or in miRNA binding sites, such as the previously mentioned SNP occurring in miR-146a, are likely to be deleterious and may have severe phenotypic consequences on human health. Therefore, extensive re-sequencing in patients and controls of 3'UTRs and of miRNAs themselves, would be definitely interesting to test the putative role of miRNA-mediated regulation in the susceptibility for anxiety disorders. Unfortunately, only 117 out of the actual 1424 miRNAs could be resequenced in this study of Quach. In this regard, the fast increase in the number of newly discovered miRNAs is being one of the main handicaps that researchers are facing. This is further complicated by the frequent corrections and modifications miRNAs suffer in their annotation (as far as sequences, nomenclature, etc).

The exponential increase in the number of miRNAs discovered was also a handicap for the design of the SNP panel used later for association analyses in disease, since the number of known miRNAs increased more than 50% from the beginning of the design to the moment that the association analyses were performed. Apart from the analyses in disease, the SNPs panel was also employed to study variability in miRNAs regions among different populations. After genotyping a group of 341 Spanish control individuals, allele frequencies between the HapMap European population and the specific North-East Spanish (Catalan) population were compared and pointed out to two genomic regions showing geographic genetic variation among populations. Remarkably, one of these regions is the LCT region (containing hsa-miR-128-1), a region that has already been shown to be under selective pressure (Beja-Pereira et al., 2003; Hollox, 2005).

Our group has been the first to show an implication of miRNAs in the aetiology of panic disorder (Table 1). Case-control studies for the 712 SNPs in the panel tagging human miRNA regions were performed in 203 Spanish patients with panic disorder and in 341 controls. Two SNPs associated with panic disorder: rs6502892 tagging miR-22 ($p<0.0002$) and rs11763020 tagging miR-339 ($p<0.00008$). Other SNPs tagging miR-138-2, miR-488, miR-491 and miR-148a regions associated with different panic disorder phenotypes, panic disorder with or without agoraphobia, or age at onset. Replication in a north-European sample of 321 anxiety patients and 653 controls from Finland and 102 patients and 829 controls from Estonia confirmed the association for several of these miRNAs. Associations alone did not result conclusive, as the associated SNPs did not resist correction for multiple testing (Muiños-Gimeno et al., 2011). Modest associations, however, are repeatedly identified in most studies on panic disorder, a disorder for which multiple genes of small effect interacting with each other and/or with non-genetic factors have been proposed to participate in disease susceptibility (Smoller and Tsuang, 1998). In fact, results on whole genome association studies suggest that susceptibility alleles are likely to be modest in effect size and require large sample sizes for detection (Sklar et al, 2008). Performing functional studies rather than replicating associations in other cohorts is gaining more attention nowadays, as recent association studies in complex disorders do not have enough power and have failed to replicate. In order to search for possible causal variants that might be in linkage disequilibrium with the associations, we re-sequenced the pre-miRNA sequences of these six associated miRNAs as well as their flanking regions. This analysis identified ten common and fourteen rare allelic variants, in addition to four short deletions, none of which

was located within the mature or pre-miRNA sequences, and therefore no effect in the targeting spectrum of the studied miRNA was predicted to occur. In contrast, effects derived from variants in the proximity of pre-miRNA sequences, if any, would be related to changes in miRNA dosage (Muiños-Gimeno et al., 2011). Indeed, variants affecting miRNA expression and processing could explain the neuronal disequilibrium proposed for psychiatric disorders, where correct dosage could be crucial.

Taking into account the fact that the targeting spectrum of the associated miRNAs was unlikely to be affected, we aimed to identify candidate genes for panic disorder, among those predicted to be targeted by the associated miRNAs, and to functionally validate these predictions. miRNA over-expression experiments using a luciferase-based assay indicated a repression of *RGS2, BDNF, HTR2C*, and *MAOA* by miR-22, of *POMC* by miR-488, of *GABRA6* by miR-138-2 and of *CCKBR* by miR-148a (Muiños-Gimeno et al., 2011). All of these genes have been implicated in the aetiology of anxiety disorders, often in a dosage dependent manner (Maron et al, 2010). For instance, serotonergic pathways have been involved in the pathogenesis of anxiety disorders, mainly because of the observation that patients with anxiety disorders respond well to serotonergic medications and because the occurrence of panic attacks has been reported after administration of serotonergic agonists (Sklar et al., 2008; Wu et al., 2008). Interestingly, the expression of *RGS2* has been demonstrated to be a quantitative trait (Betel et al., 2008), for which association with a haplotype within the 3'UTR has been reported (Maron et al., 2010; Koene et al., 2009). Moreover, *RGS2* knock-out mice show increased anxiety-like behaviour compared to their wild-type counterparts (Yalcin et al., 2004), remarkably, the expression of *RGS2* was significantly reduced in experiments where over-expression of miR-22 was simulated in neuroblastoma SH-SY5Y cells (Muiños-Gimeno et al., 2011). Interestingly, another down-regulated gene after miR-22 over-expression was *ASCL1*, which has been demonstrated to be essential for the development of central serotonergic neurons and has been proposed as a candidate for Ondine syndrome, a rare disorder of the chemical control of breathing (Pattyn et al, 2004, de Pontual et al., 2004). Other down-regulated genes also have important neuronal functions, such as *CHGA* with roles in neuroendocrine secretion (Taupenot et al., 2003) or the promotion of dendritic outgrouth by *NPTX2* (Tsui et al., 1996). Furthermore, it is worth remarking the deregulation of the corticotropin releasing hormone (CRH) signalling pathway associated with the over-expression of miR-488 in the same cellular system, this is a crucial pathway activated in response to stress and includes the pro-opiomelanocortin (*POMC*) gene. POMC is the precursor molecule for several important components of the hypothalamic-pituitary-adrenal axis (Figure 1), which is involved in the neurobiology of mood and anxiety disorders (Swaab et al., 2005). Regardless of the genetic mechanism involved in these associations, the development of the phenotype could depend upon the expression and activity of these miRNAs, some of which are known to be expressed or to have important functions in the brain. miR-22, for example, is expressed ubiquitously in several tissues including the pituitary and the midbrain; miR-488 is a brain-enriched miRNA that is more abundantly expressed in the hippocampus and cerebellum; miR-138 is highly enriched in the brain, including the frontal cortex, the hippocampus and the midbrain (Betel et al., 2008). Furthermore, miR-138 is localized within dendrites, and is known to negatively regulate the size of dendritic spines in rat hippocampal neurons (Siegel et al., 2009)

Finally, it is important to mention two studies that, using animal models, involve particular miRNAs with anxiety behaviour. The diversity of phenotypes available for several mouse strains has allowed to determine differences in miRNA expression across inbred strains and

to analyze their correlation with both phenotype data and mRNA regulation (Parsons et al., 2008). Following this approach the authors managed to nominate miRNAs that have potential roles in anxiety, exploration, and learning and memory. In particular, they found correlation between the differential expression of miR-34c and mir-323 with behavioural measures for anxiety. It is interesting to highlight that the authors also suggest a miR-34c-mediated regulation of genes involved in long-term depression and neuroactive ligand receptors. This study has opened the door for further research using mouse genetic reference populations and importantly points out to two miRNAs, miR-34c and miR-323, as candidate genes for anxiety disorders (Parsons et al., 2008). Another recent study working with Fischer 344 rats, as an animal model for the study of vulnerability to repeated stress -and therefore for anxiety and mood disorders-, described a role for miR-18a in the regulation of hypothalamic-pituitary-adrenal axis. Suppressed or decreased hypothalamic-pituitary-adrenal axis responses have been repeatedly described on chronically stressed animals upon re-exposure to the same stressor. This phenomenon, called habituation, is likely to protect the organism from hypercoticosteroidism and is thought to be partly controlled via activation of glucocorticoid type I (mineralocorticoid) and/or glucocorticoid type II (glucocorticoid receptor). Interestingly, Fischer 344 rats have been reported to exhibit no habituation of hypothalamic-pituitary-adrenal axis activity during restraint stress episodes. The study of Shusaku *et al*. reported an increased expression of miR-18a and lower glucocorticoid receptor protein levels in the hypothalamic paraventricular nucleous of Fischer 344 rats. This along with the fact that miR-18a seems to inhibit glucocorticoid receptor translation (Figure 1) suggests that miR-18-a could be a vulnerability factor for the development of stress-related behaviours (Shusaku et al., 2008). Consequently, miR-18a together with miR-34 and miR-323 should be considered as potential susceptibility factors for stress-related disorders such as panic and mood disorders (Table 1).

5.2 Trans-deregulation of miRNAs

Trans-deregulation of miRNAs can be broadly summarized into structural alterations involving genes that are important in miRNA biogenesis (e.g Fragile X and DiGeorge syndromes), or mutations in miRNAs target mRNA sequences (e.g. Tourette syndrome and pathological aggressiveness). A few examples of human diseases affecting the central nervous system caused by deregulation in the miRNA pathway have been reported, such as cancer or Fragile X Syndrome (Gong et al., 2005). Fragile X syndrome (FX) is one of the most common forms of mental retardation, and is characterized by abnormalities in the structural development of dendritic spines. It is caused by a CGG repeat in the 5'UTR of the *FMR1* gene, which is located on the long arm of chromosome X. The condition becomes clinically manifest when the repeat expands as the gene is passed from generation to generation, until its transcription is completely shut down in the full-blown syndrome (Penagarikano et al., 2007). The current view is that Fragile X protein (FMRP) associates with endogenous miRNAs and with Ago1 -in mammals -to translationally repress a subset of dendritic mRNAs (Jin et al., 2004) and that the disease is caused by the deregulated expression of its mRNA targets which encode factors required for synaptic plasticity and development. In relation to this, another study carried out in Drosophila has shown an interesting overlapping between the composition of FMRP-containing neuronal granules and P-bodies, which suggests that these classes of granules might be similar not only in composition but also in function (Hillebrand et al., 2007). Similarly, most cases of DiGeorge syndrome

miRNA	Involvement with Anxiety Disorder	Reference
miR-18a	Possible repressor of the glucorcorticoid receptor gene in the hypothalamic paraventricular nucleus regulating stress responses	Shusaku et al., 2008
miR-34c	Correlation between differential expression of this miRNA and behavioural measures for anxiety in mice	Parsons et al., 2008
miR-323	Correlation between differential expression of this miRNA and behavioural measures for anxiety in mice	Parsons et al., 2008
miR-128	Association of an allelic variant in the target site for miR-128 in *NTRK3 (ss102661458)* with Panic Disorder - Reduction of *NTRK3* repression	Muiños-Gimeno et al., 2009
miR-509	Association of an allelic variant in the target site for miR-509 in *NTRK3 (ss102661458)* with Panic Disorder - Reduction of *NTRK3* repression	Muiños-Gimeno et al., 2009
miR-765	Association of an allelic variant in the target site for miR-765 in *NTRK3 (ss102661460)* with Panic Disorder - Reduction of *NTRK3* repression	Muiños-Gimeno et al., 2009
miR-485-3p	Association of an allelic variant in the target site for miR-765 in *NTRK3* (rs28521337) with Obsessive-Compulsive Disorder	Muiños-Gimeno et al., 2009
miR-22	Associated with Panic Disorder - Repression of *RGS2*, *BDNF*, *HTR2C*, and *MAOA*	Muiños-Gimeno et al., 2011
miR-138-2	Associated with age at onset in Panic Disorder - Repression of *GABRA6*	Muiños-Gimeno et al., 2011
miR-148a	Associated with Panic Disorder - Repression of *CCKBR*	Muiños-Gimeno et al., 2011
miR-339	Associated with Panic Disorder	Muiños-Gimeno et al., 2011
miR-488	Associated with Panic Disorder - Repression of *POMC*	Muiños-Gimeno et al., 2011
miR-491	Associated with Panic Disorder	Muiños-Gimeno et al., 2011

NTRK3, neurotrophic tyrosine kinase, receptor, type 3; *RGS2*, regulator of G protein signaling 2; *BDNF*, brain-derived neurotrophic factor; *HTR2C*, 5-hydroxytryptamine (serotonin) receptor 2C; *MAOA*, monoamine oxidase A; *GABRA6*, gamma-aminobutyric acid A receptor, alpha 6; *CCKBR*, cholecystokinin B receptor; *POMC*, proopiomelanocortin preproprotein.

Table 1. Overview of miRNAs reported to be involved in anxiety disorders.

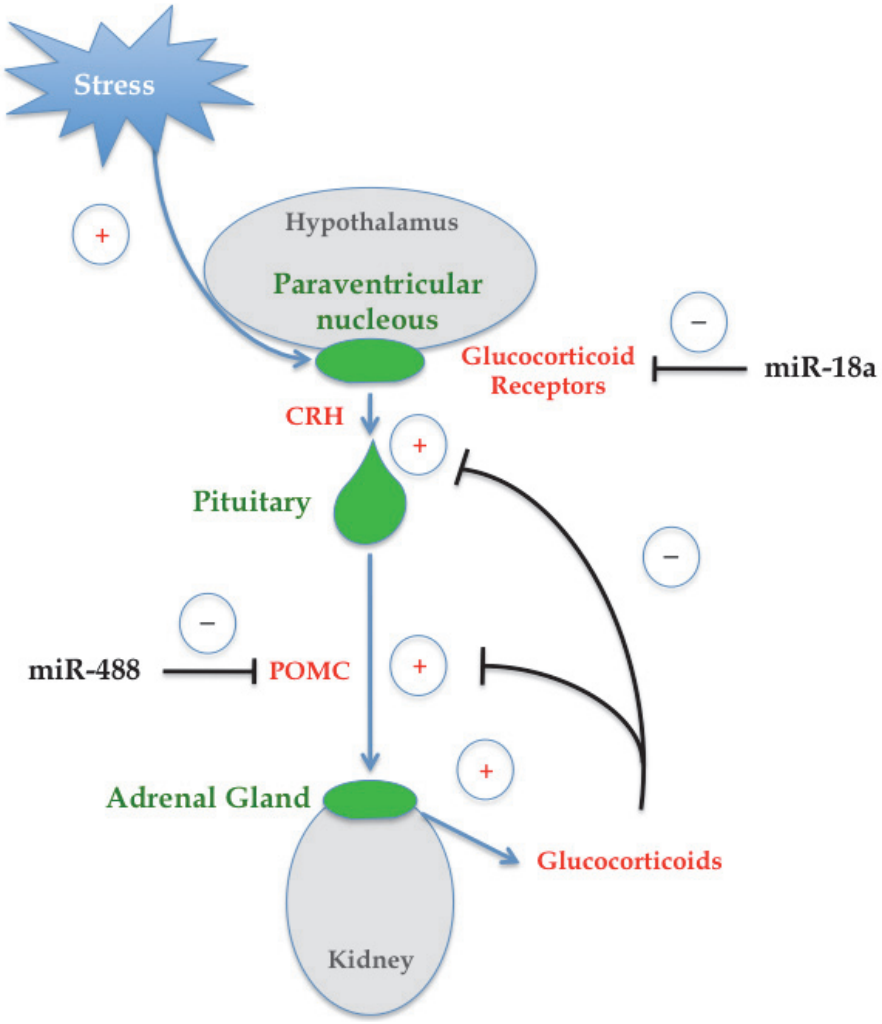

Fig. 1. Overview of the hypothalamic-pituitary-adrenal axis activation in response to stress showing the putative regulation of the pathway at two different points by miR-18a and miR-488. CRH, corticotropin releasing hormone ; POMC, pro-opiomelanocortin.

result from a deletion of chromosome 22q11.2 (the DiGeorge syndrome chromosome region, or DGCR); this deletion includes the *DGCR8* gene. DiGeorge syndrome is a developmental disorder characterized by mental retardation, as well as structural and functional palate anomalies, conotruncal cardiac malformations, immunodeficiency, hypocalcemia, and typical facial anomalies. DGCR8 is required for the maturation of miRNA primary transcripts. In fact, its knockdown leads to accumulation of pri-miRNAs and reduction of pre-miRNAs and mature miRNAs (Landthaler et al., 2004), indicating again a plausible involvement of miRNAs in the aetiology of the disease.

Since 2005 until now, the number of described mutations in miRNAs target mRNA sequences (poly-miRTS) has been growing exponentially, being the majority of these studies published in the last three years. On this section we will only focus on those poly-miRTS affecting central nervous system; nevertheless, poly-miRTS have also been implicated in different disorders such as breast cancer (Adams et al., 2007), hypertension (Martin et al., 2007; Sethupathy et al., 2007), methotrexate resistance (Mishra et al., 2007), Childhood asthma (Tan et al., 2007) and colorectal cancer (Landi et al., 2008) among others. Abelson *et al.* were the first to associate a sequence variant in a miRNA target site with disease in 2005 (Abelson et al., 2005). They reported a rare sequence variant enhancing a target site for miR-189 in the *SLITRK1* (Slit and TRK-like family member 1) gene in two patients with Tourette's syndrome and in none of the controls tested. An altered interaction between this miRNA and the *SLITRK1* mRNA in the developing brain was suggested to contribute to this neuropsychiatric disorder. However this study has been treated with scepticism since a follow-up study indicated that the variant associated with Tourette Syndrome was overrepresented in certain subgroups such as Ashkenazi Jews, thereby complicating the interpretation of the results in a sample where cases and controls could happen to be not appropriately matched (Keen-Kim et al., 2006). After that, the putative importance of poly-miRTS was strengthened by other studies involving SNPs affecting miRNA target sites with disease. Regarding those affecting the central nervous system two mutations were identified in the 3'UTR of the receptor expression enhancing protein 1 gene (*REEP1*), after previous association of this region with hereditary spastic paraplegia (Zuchner et al., 2006). These mutations were predicted to strengthen miR-140 mediated repression. Independently, in a similar approach, Beetz *et al.* identified one of these variants and discovered a third variant putatively affecting miR-691 regulation in two other hereditary spastic paraplegia families (Beetz et al., 2008). However, in these studies no functional experiments were performed. Jensen *et al.*, aimed to study the role of miRNAs in human behaviours; as the deletion of serotonin receptor 1B (*HTR1B*) has been shown to cause aggressive behaviour in mice, the authors analyzed the gene's 3'UTR. A common polymorphism in the serotonin receptor 1B affecting the target site for miR-96 was shown to be associated with aggressiveness in humans (Jensen et al., 2008). Finally, a polymorphism in fibroblast growth factor 20 (*FGF20*) conferring a risk to Parkinson's disease was shown to disrupt a miR-433 target site. Unlike other studies, the authors went further to provide human in vivo validation of this target site and a molecular mechanism by which differential miR-433 targeting leads to Parkinson's disease. According to their report, disruption of miR-433 target site leads to increased *FGF20* translation, which in turn, increases alpha-synuclein expression and ultimately causes Parkinson's disease (Wang et al., 2008b).

5.2.1 Trans-deregulation of miRNAs and anxiety disorder

Under the same hypothesis, the possible implication in anxiety disorders of genetic variants affecting miRNA-mediated regulation of candidate genes has been already studied in the particular case of the neurotrophic tyrosine kinase 3 gene, *NTRK3*. Based on the recent discovery of miRNA-mediated regulation for different isoforms of this gene (Laneve et al., 2007; Guidi et al, 2010) *NTRK3* was analyzed as a predisposition factor for anxiety disorders by re-sequencing the different 3'UTRs of two different isoforms of the gene (Muiños-Gimeno et al., 2009). The study led to the identification of several variants in the 3'UTR of the truncated isoform of this gene (Table 1). Remarkably, one common SNP (rs28521337)

was found to be associated with obsessive-compulsive disorder. Nevertheless, this association did not resist correction for multiple testing in the obsessive-compulsive disorder sample and was only statistically significant for the hoarding sub-clinical type of this disorder, suggesting a different pattern of genetic inheritance for this group of patients, which would be in agreement with recent reports that indicate that hoarding subphenotype may constitute a neurobiologically and etiologically distinct variant of obsessive-compulsive disorder (Miguel et al., 2005; Samuels et al., 2007), being highly heritable as a quantitative trait (Mathews et al., 2007). In the same study, other two rare variants in the 3'UTR of the truncated isoform of *NTRK3* were identified, one of them is located in the target site and, specifically, in the sequence that binds to the seed region of miR-765 and the second variant in the target site for two different miRNAs, miR-509 and miR-128, the latter being a brain-enriched miRNA involved in neuronal differentiation and synaptic processing. Interestingly, after mutagenesis and functional analyses of these two variants, both of them were shown to cause a significant alteration in the miRNA-mediated regulation of *NTRK3* resulting in the recovery of gene expression when compared with the control sequence (Muiños-Gimeno et al., 2009). On the other hand, the contribution of rs28521337 to the susceptibility to obsessive-compulsive disorder remains unclear because, although the variant is located in the seed region of a validated target site for miR-485-3p, it did not significantly change the affinity and efficiency of this miRNA. Albeit it is possible that rs28521337 is only in linkage disequilibrium with the real cause of the disorder, the possibility that this SNP might be altering the expression of *NTRK3* in a miRNA independent mechanism or that HeLa cells, the cell type used for the functional study, lack the additional cofactors required for the release of miRNA mediated repression of *NTRK3* cannot be excluded. In this sense, it would be interesting to analyze the functional consequences of this SNP in a more biologically relevant context.

On the other hand, contribution to disease of the two rare allelic variants at the population level remains low, due to the fact that these variants were only identified in one male patient with panic disorder and agoraphobia, each. While it is widely accepted that rare allelic variants contribute little to heterozygosity, their putative role on disease cannot be ruled out as emphasized by the results on recent whole genome association studies, which have failed to identify major alleles for most of the disorders studied. These studies point out that susceptibility alleles are likely to be modest in effect size requiring large samples for detection (Sklar et al., 2008). Similarly, the rare allelic variant in the 3'UTR of *SLITRK1* affecting the binding of miR-189 was only identified in two patients with Tourette syndrome and in none of the controls tested (Abelson et al., 2005). However, as previously mentioned, other groups (Keen-Kim et al. 2006) were unable to replicate the study and identified this rare allelic variant among cases and controls within the same families. In their opinion these results indicate that the variant does not segregate with the disease and that the results from Abelson *et al.* might be confounded by hidden population stratification (Keen-Kim et al., 2006). Confounding might originate on the fact that the screening for new causative allelic variants was carried out only in cases. Nevertheless, that the rare variant did not segregate completely with the disease may be explained, at least partly, by the difficulty, that the study of psychiatric disorders encompasses. In Tourette syndrome, as in anxiety disorders, diagnoses may not always be correctly assessed as most cases are mild and may remain undiagnosed. Moreover, genetic and environmental factors are likely to be involved in their aetiology and, consequently, among family members of an affected person, it is difficult to

predict who else may be at risk of developing the condition, in other words, their inheritance patterns are unclear. This fact underlies a heterogenic basis of the disorder, in which cases substantially differ from one another, even though they are considered to suffer from the same disease. Thus, most probably, environmental factors, genetic epistasis and/or accumulation of rare variants might be on the back of the susceptibility to these disorders. Consistently, the case-control studies that we performed with the SNP panel covering miRNA regions in panic disorder resulted on moderate associations for several miRNA regions and, in addition, increase of risk in most of the cases was in general moderate as indicated by Odds Ratios and Confidence Intervals. These findings are therefore in line with the genetic heterogeneity theory proposed for anxiety disorders.

Other interesting methods used for the study of anxiety are based on a cross-species approach addressed to identify genes that regulate anxiety-like behaviour. Using this kind of approach, a SNP in the 3'UTR of the aminolevulinate dehydratase (*ALAD*) was found to be associated with social phobia. Even though this is not a true poly-miRTS study, this association is of particular interest since the authors comment on the possibility that this SNP generates an illegitimate target site for miR-211 and miR-204 -as predicted by a miRNA target prediction program- (Donner et al., 2008). In this regard, an important bottle-neck in the study of poly-miRTS is the identification of miRNA target sites itself. As part of the effort to understand interactions between miRNAs and their targets, computational algorithms have been developed based on observed rules for features, such as the degree of hybridization between the two RNA molecules. These *in silico* approaches provide important tools for miRNA target detection and, together with experimental validation, help to reveal miRNA target genes.

6. Conclusions

Anxiety disorders have long been believed to have abnormal neural regulatory mechanisms underlying symptom manifestation. We here propose that this deregulation is mediated, at least partially, by defective miRNA action, that consequently derives in dosage changes of proteins involved in central nervous system function. Mutations in miRNA target sites, such as those found in the *NTRK3* gene, that might be affecting miRNA regulation, as well as anomalous dosage of miRNAs themselves, as observed for miR-18a, could be responsible for a disruption in the accurate equilibrium of complex brain functions, contributing to the development of anxiety disorders. In fact, miRNA expression differences between mice strains have been already showed to play a significant role in mice behaviour and have pinpointed to miR-34c and miR-323 as potential candidate genes for anxiety disorders. Moreover, the finding of at least four miRNAs (miR-22, miR-138-2, miR-148a and miR-488) associated with panic disorder that repress genes that have been previously involved in anxiety disorders, namely, *NTRK3, RGS2, GABRA6, CCKBR, BDNF, HTR2C* and *MAOA,* provides important new evidence that variation in genes coding for miRNAs may mis-coordinate a number of risk genes and thereby contribute to the development of panic disorder. Taken together these data demonstrate the importance that alterations in the complex circuitry of gene regulation, in which miRNAs are involved, may have, not only in the fine functioning of the human central nervous system, but also in other physiological pathways linked to the development of stress-related disorders, further sustaining the hypothesis that miRNA-induced differential dosage may be participating in the aetiology of anxiety disorders.

7. Future perspectives

Overall, the genetic complexity observed for anxiety disorders, where multiple alleles -rare or common- in protein coding genes or in regulatory elements might contribute independently to marginal increases of risk, together with the fact that these disorders are also influenced by environmental factors, evidences that traditional association analyses are probably underestimating the contribution of an analyzed locus to the studied disorder. In fact, methods based on linkage disequilibrium such as case-control analyses -using candidate genes or whole genome studies- have shown little success in identifying causative variants for anxiety disorders. Hence, the use of new high-throughput sequencing technologies and an increase of sample sizes would be of great help to dissect the underlying molecular causes of these complex disorders. In fact, the improvement and price reduction of these technologies are starting to make this type of analyses feasible nowadays. On the other hand, the recent and increasing evidence that supports an important role for regulatory regions in shaping phenotypes that, in last term, might be related to disease, makes the inclusion of these elements in future analyses of every single disorder of crucial importance. This is particularly true in the case of miRNAs; the rapid growth in the discovery of functional miRNA targets, as well as the involvement of new miRNA-related mechanisms in disease must be accompanied by an improvement in the tools needed to explore this new miRNA world. The latter should include the generation of proficient prediction algorithms and validation tools for the identification of miRNA target genes as well as generation of computational and experimental approaches to better understand how polymorphisms might be affecting pre-miRNA transcription, structure and mature miRNA expression and processing. In this sense, once potential causative allelic variants are identified, their contribution to disease remains always controversial and may only be assessed by means of functional experiments that demonstrate their possible involvement in the disorder. Accordingly, implementation of new functional approaches, as the ones presented along the chapter, are needed to identify candidate biological pathways involved in anxiety. We would finally like to encourage the current view that underlines the importance of converging data from genetics, analysis of cognitive function, study of animal models and neuroimaging in order to achieve a more integrative picture of complex human disorders in the near future.

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16

Neurosteroid Biosynthesis Upregulation: A Novel Promising Therapy for Anxiety Disorders and PTSD

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1. Introduction

Generalized anxiety, panic, and posttraumatic stress disorder (PTSD) are debilitating conditions, which have an incidence of one in ten persons in the general population and epidemiological studies also report that these disorders often occur with depression (1-3). Anxiolytic benzodiazepines, including diazepam and alprazolam, remain the best and most used treatments for these conditions (4-7). However, their therapeutic use is associated with side effects, which include sedation and rapid development of tolerance as well as dependence. This results in severe discontinuation symptoms and often to drug abuse (4-6, 8; 9).

In many patients, including patients with PTSD, the pharmacological effects of these drugs are very weak and there is a large number of non-responders (10-12). This has stimulated drug design that for many decades has focused in the development of new more effective therapies for anxiety disorders (13-15). Novel neuronal biomarkers for the pharmacological targets of the next generation of anxiolytic drugs have been discovered.

The downregulation of neurosteroid biosynthesis has been implicated in the pathophysiology of anxiety and depressive disorders (reviewed in 16). Decreases in cerebrospinal fluid (clinical studies) and brain content (preclinical studies) of the $GABA_A$ receptor-active progesterone derivative, allopregnanolone, have been associated with affective and mood disorders, which includes depression, anxiety spectrum disorders, PTSD, premenstrual dysphoric disorder, schizophrenia, and impulsivity (17-27). Thus, elevating or normalizing the downregulation of brain allopregnanolone levels could be a promising therapeutic strategy for these psychiatric disorders. This prompted investigations to develop new neurosteroidogenic agents to contrast allopregnanolone biosynthesis deficits in anxiety and depression (28-31).

We measured allopregnanolone levels in the cerebrospinal fluid (CSF) of PTSD patients assuming that allopregnanolone levels in the CSF reflect the levels of this neurosteroid in the brain (17). Also, in depressed patients, the concentration of allopregnanolone in the CSF was decreased by about 50-60% of the levels measured in non-psychiatric patients (26). The CSF allopregnanolone level decrease is likely induced by a downregulation of the expression of 5α-reductase type I mRNA in the prefrontal-cortex (area BA9) that we measured in

depressed patients and age- and sex-matched non-psychiatric subjects (32). The cortical level of 5α-reductase mRNA in depressed patients was dramatically decreased to about 50% of the levels measured in non-psychiatric comparison subjects, whereas the levels of 5α-reductase mRNA was unchanged in the cerebellum (32). In depressed patients, SSRI treatment with fluoxetine and fluvoxamine normalized the CSF allopregnanolone content (26) in a manner that correlated with the improvement in depressive symptoms. These results were confirmed in studies that determined allopregnanolone or levels of 5α-tetrahydrodeoxycorticosterone, another positive modulator of $GABA_A$ receptor function, in the plasma of depressed patients treated with SSRIs (33).

In premenopausal women with PTSD, the CSF allopregnanolone levels were decreased by about 60% and were inversely correlated with PTSD re-experiencing and comorbid depressive symptoms (17). Interestingly, CSF allopregnanolone levels were lowest in those patients with PTSD and comorbid depression. Also, the *ratio* of allopregnanolone to its steroid precursor, 5α-dihydroprogesterone (5α-DHP), was decreased among the PTSD patients, suggesting the presence of an impairment in the biosynthesis of allopregnanolone from its precursor 5α-DHP (17). These data suggest that the downregulation of brain allopregnanolone levels in PTSD and depressed patients may cause a GABAergic neurotransmission dysfunction, which in turn results in the behavioral symptoms seen in these patients.

Following the finding that fluoxetine and paroxetine and other SSRIs increase the content of allopregnanolone in several rodent brain structures (34), we hypothesized that normalization of brain allopregnanolone levels may underlie the pharmacological effects of the so called "selective serotonin reuptake inhibitors" or SSRIs in mood disorders. To test this hypothesis, we conducted experiments using the socially isolated mouse as an animal model of anxiety disorders and PTSD (16; 35-38). The socially isolated mouse expresses a robust decrease of corticolimbic allopregnanolone levels, which are associated with anxiety-like behaviors, fear, resistance to sedation, and heightened aggression (16; 35; 39). These behavioral deficits can be ameliorated by administration of fluoxetine and other SSRIs that upregulate allopregnanolone levels. Interestingly, fluoxetine's pharmacological effects resulted to be independent from the ability of this drug to inhibit serotonin reuptake (35; 36).

Our experiments support a selective and novel mechanism whereby SSRIs, acting as selective brain *steroidogenic* stimulants (SBSSs), increase brain corticolimbic allopregnanolone levels and improve PTSD, anxiety, and depression behavioral symptoms.

2. Neurosteroids modulation of $GABA_A$ receptors function

Biosynthesis of neurosteroids in the brain is independent from adrenals, ovaries, and testis (40-44). Neurosteroids are functionally active in modulating gene expression and neurotransmitter systems (45-52). Allopregnanolone exerts pharmacological actions, such as anticonvulsant, anxiolytic, antidepressant, and even sedative-hypnotic (53-60). These pharmacological actions are similar to those elicited by barbiturates and benzodiazepines (52; 61; 62). Allopregnanolone potently (nM affinity), positively, and allosterically modulates the action of GABA at $GABA_A$ receptors (45-51). The endogenous physiological relevance of allopregnanolone is substantiated by its facilitation and fine-tuning of the efficacy of direct $GABA_A$ receptor activators and positive allosteric modulators of GABA action at $GABA_A$ receptors (43; 47; 48; 63). The demonstration that allopregnanolone potentiates GABA responses via two binding sites in the $GABA_A$ receptor that, respectively, mediate the potentiation and the direct activation of the $GABA_A$ receptor by allopregnanolone has been

pivotal in neurosteroid pharmacology (64). Also, $GABA_A$ receptors incorporating α4, α6, and δ subunits in combination with γ and β subunits show higher affinity (nM range) for allopregnanolone (45; 46; 51; 64; 65). Relevant for pharmacological strategies to overcome behavioral deficits resulting from $GABA_A$ receptor signal transduction deficits, allopregnanolone allosteric modulation of the action of GABA at $GABA_A$ receptors is much less selective than that of benzodiazepines, which are relatively inactive at α4- or α6-containing $GABA_A$ receptors (4; 45; 46; 66).

3. Neurosteroid biosynthesis in corticolimbic neurons

A study of the neuronal localization of the neurosteroidogenic enzymes, 5α-reductase type I and 3α-hydroxysteroid dehydrogenase (3α-HSD), has recently showed that these enzymes are not expressed in GABAergic cortical interneurons or glial cells (67). Of note, 5α-reductase and 3α-HSD were highly expressed and co-localized in a region-specific way in primary GABAergic and glutamatergic neurons, including pyramidal neurons, granular cells, reticulo-thalamic neurons, medium spiny neurons of the striatum and nucleus accumbens, and Purkinje cells in the cerebellum (67). This suggested that allopregnanolone synthesized in glutamatergic cortical or hippocampal pyramidal neurons or in granular cells of the dentate gyrus may be secreted in: 1) a paracrine manner which would allow allopregnanolone to reach $GABA_A$ receptors located in the synaptic membranes of other cortical or hippocampal pyramidal neurons, or 2) an autocrine fashion which would allow allopregnanolone to act locally by binding post-synaptic or extra-synaptic $GABA_A$ receptors located on the same dendrites or cell bodies of the cortical or hippocampal pyramidal neuron in which it was produced (67). Alternatively, allopregnanolone might not be released, but may instead diffuse laterally into synaptosome membranes of the cell bodies or dendritic arborization of glutamatergic neurons in which it is produced to attain intracellular access to specific neurosteroid binding sites of $GABA_A$ receptors (67; 68). In the amygdala, for example, this would functionally baffle the effects of concomitant excitatory inputs to glutamatergic projection neurons during exposure to unconditioned stress during fear conditioning or to conditioned stressors during extinction.

On the other hand, allopregnanolone produced in primary output GABAergic neurons from the reticular thalamic nucleus may secrete allopregnanolone simultaneously with GABA to concomitantly act at post-synaptic $GABA_A$ receptors inserted in glutamatergic thalamocortical neurons (69). Very similarly, allopregnanolone synthesized by striatal medium spiny GABAergic neurons and cerebellar Purkinje cells may activate post-synaptic $GABA_A$ receptors located on cell bodies or dendrites of neurons in the deep cerebellar nuclei (67).

The clarification of allopregnanolone site of synthesis and action across several brain regions has been pivotal to our understanding of the possible mechanisms by which allopregnanolone is secreted and acts at $GABA_A$ receptors. These studies underscore the functional role of allopregnanolone in fine tuning the strength of GABAergic neurotransmission under physiological conditions and how deficits in allopregnanolone biosynthesis may result in abnormal behavior.

4. Social isolation induces a selective neuron-specific decrease of 5α-reductase in corticolimbic neurons

Exposure of rodents to protracted social isolation stress for 4-8 weeks induces a decrease in allopregnanolone biosynthesis in several corticolimbic structures as a result of a

downregulation of the mRNA and protein expression of 5α-reductase type I (35; 70-73; reviewed in 38). Socially isolated mice show a 70% reduction in the synthesis rate of allopregnanolone and 5α-DHP biosynthesis compared to group-housed mice (35; 72).
Allopregnanolone and 5α-DHP are unevenly distributed and expressed in various brain structures (48; 74). The rodent olfactory bulb shows the highest concentrations of 5α-DHP and allopregnanolone followed by the frontal cortex, hippocampus, amygdala, striatum, and cerebellum (74). Interestingly, the largest decrease of 5α-reductase was found in brain regions regulating emotional behavior, including the amygdala and hippocampus, followed by the olfactory bulb and the frontal cortex (74). The expression of 5α-reductase failed to change in the cerebellum and striatum (74; 75). Decreased 5α-reductase was specifically found in cortical pyramidal neurons of layers V-VI, in hippocampal CA3 pyramidal neurons and glutamatergic granular cells of the dentate gyrus, and in the pyramidal-like neurons of the basolateral amygdala (75). However, 5α-reductase fails to change in GABAergic neurons of the reticular thalamic nucleus, central amygdala, cerebellum, and in the medium spiny neurons of the caudatus and putamen (75). In these brain areas, we confirmed that the decrease of 5α-reductase resulted in a reduction of allopregnanolone levels (74; 76; 77). Social isolation failed to change the expression of 3α-HSD, the mRNA expression of diazepam binding inhibitor, and the expression of the 18 kDa translocase protein (TSPO), which is involved in the transport of cholesterol across the inner mitochondrial membrane and activation of neurosteroidogenesis (reviewed in 72). Thus, the downregulation of 5α-reductase appears to be the main factor responsible for the reduction of corticolimbic allopregnanolone levels.

5. GABAergic neurotransmission deficits resulting from allopregnanolone downregulation

Allopregnanolone biosynthesis downregulation as a result of social isolation stress or pharmacological decrease of allopregnanolone induced by inhibiting 5α-reductase with the potent competitive 5α-reductase inhibitor SKF 105,111 decreases GABAergic neurotransmission as demonstrated by reduced loss of righting reflexes induced by $GABA_A$ receptor active ligands. The effects of SKF on the muscimol-, pentobarbital-, benzodiazepine-, or alcohol-induced loss of righting reflex loss can be reversed by the systemic or intracerebroventricular administration of allopregnanolone (43; 48). Likewise, social isolation or SKF-induced decrease of allopregnanolone results in facilitation of the seizure activity induced by several drugs that decrease $GABA_A$ receptor function, including picrotoxin (63). Administration of allopregnanolone at doses that have virtually no effects on group-housed control mice normalized the increased susceptibility to picrotoxin-induced seizures in SKF-treated or social isolated mice (63). The protracted social isolation or SKF treatment-induced allopregnanolone biosynthesis downregulation appeared to be the primary reason for the $GABA_A$ receptor signal transduction deficits observed in these mice. In fact, seizures induced by kainic acid or strychnine in socially isolated mice are similar to those induced by these agents in group housed mice.

6. Behavioral effects induced by allopregnanolone downregulation in corticolimbic areas

The decrease of allopregnanolone biosynthesis in socially isolated mice has been associated with several behavioral deficits that resemble behavioral abnormalities observed in patients

with PTSD (16; 17; 30; 38). Hence, this mouse model can be used to study the behavioral responses elicited by treatment with neurosteroidogenic agents, the SBSSs. This new class of drugs includes the SSRI antidepressants that have been shown to elicit a potent neurosteroidogenic activity selectively at low doses as their principal action.

Allopregnanolone has emerged as an important biomarker of emotional behavioral deficits (16; 35-38; 72). This was demonstrated by experiments using socially isolated mice to induce a downregulation of allopregnanolone biosynthesis. We have established a fundamental role for allopregnanolone in the regulation of anxiety-like and aggressive behavior as well as contextual fear conditioning, (16; 37; 63; 74; 77). When mice are socially isolated for a period varying from one to eight weeks, there is a time-dependent increase in aggressive behavior over the first four weeks of isolation, which is inversely correlated with a time-dependent decrease of corticolimbic allopregnanolone levels (35). Likewise, socially isolated mice exposed to a classical fear conditioning paradigm showed enhanced conditioned contextual but not cued fear responses compared with group housed mice (74). The time-related increase of contextual fear responses correlated with the downregulation of 5α-reductase mRNA and protein expression observed in the frontal cortex, hippocampus, and amygdala (74). Socially isolated mice also exhibited impaired and incomplete fear extinction (74). Of note, socially isolated mice also exhibit higher levels of anxiety-like behavior, determined by the elevated plus maze and in the open field (16; 39).

Allopregnanolone plays a *pivotal* rather than incidental role in the regulation of contextual fear responses and aggression. In fact, pharmacological treatment with allopregnanolone dose-dependently decreased aggression in a manner that correlated with an increase in corticolimbic allopregnanolone content (35). Allopregnanolone also normalized the exaggerated contextual fear responses and anxiety of socially isolated mice (74). Further, administration of the potent 5α-reductase competitive inhibitor SKF 105,111 to normal group-housed mice (43; 48; 47) rapidly (~1 h) decreased levels of allopregnanolone in the olfactory bulb, frontal cortex, hippocampus, and amygdala by 80-90% (73; 74) in association with a dose-dependent increase of conditioned contextual fear responses (74). Administering allopregnanolone doses that normalized hippocampus allopregnanolone levels reversed the effects of SKF 105,111 on conditioned contextual fear responses (74). These results are in agreement with results of many other investigators who have observed that allopregnanolone elicits anxiolytic and antidepressant effects (39; 54; 78-84).

7. Social isolation induces changes in $GABA_A$ receptor subunit expression

Postmortem studies suggest that altered corticolimbic GABAergic neurotransmission, GABA receptor binding and receptor subunit composition, as well as GABA synthesis and transport may be associated with various psychiatric disorders, including anxiety disorders, schizophrenia, and depression (85-88).

The regional distribution of $GABA_A$ receptor subunit subtypes plays an important role in the pharmacology of $GABA_A$ receptor ligands that bind to selective and specific $GABA_A$ receptor subunits (89-90). Recent studies showed that α1-containing $GABA_A$ receptors mediate the sedative properties of specific GABAergic ligands, such as diazepam, in the same way α2 and probably α3 subunits mediate the anxiolytic effects of benzodiazepines, and α5 subunits appear to be involved in learning and cognition (89; 90). High affinity binding of benzodiazepine to $GABA_A$ receptors requires the interaction of α and γ subunits (89; 90).

In socially isolated mice, we found changes in the mRNA and protein expression of several $GABA_A$ receptor subunits in the frontal cortex and hippocampus (91). The mRNA levels encoding α1, α2, and γ2 $GABA_A$ receptor subunit subtypes were reduced (~50%), while the mRNAs encoding α4 and α5 subunits were increased (~130%) compared to levels measured in group-housed mice (91). Protein levels of α1 and α5 determined in synaptic membrane preparations in the frontal cortex and hippocampus confirmed the former results. Using a laser microdissection technique coupled with nested RT-PCR amplification, we found that α1 mRNA levels were decreased by 50% in layer I neuropil, whereas the expression of α1 subunit mRNA in the pyramidal neurons of layer V was unchanged as a result of social isolation. Thus, changes in $GABA_A$ receptor subunits within one brain area are region-specific (91).

Changes in $GABA_A$ receptor subunit subtype composition are expected to result in altered pharmacological responses to various $GABA_A$ receptor ligands in socially isolated mice. As expected, socially isolated mice showed resistance to the sedative and anxiolytic properties of diazepam and zolpidem, positive allosteric $GABA_A$ receptor modulators that bind with high affinity to α1, α2, α3 or α5 subunit-containing $GABA_A$ receptors (diazepam) and to α1 subunit-containing $GABA_A$ receptors (zolpidem) (91). The α1 subunit of the $GABA_A$ receptor plays a primary role in mediating the sedative pharmacological effects of diazepam and zolpidem (92). Hence, their altered pharmacological response could result by a decrease in α1 subunit-containing $GABA_A$ receptors. Likewise, a decreased γ2 subunits support the formation of $GABA_A$ receptors in which this subunit might be substituted. Given that γ2 subunits are a necessary prerequisite for the formation of benzodiazepine-sensitive $GABA_A$ receptors (89; 90), the lack of anxiolytic activity of diazepam may result from the formation of benzodiazepine-insensitive $GABA_A$ receptors in neuronal circuits that regulate anxiety (39; 91).

Increases of α4 subunit-containing $GABA_A$ receptor expression in the frontal cortex appeared to be irrelevant to the behavioral or pharmacological alterations observed in socially isolated mice. GABA agonists such as THIP or the allosteric modulator, allopregnanolone, show selectivity and increased potency, respectively, for $GABA_A$ receptors containing α4/δ-subunits. These compounds comparably decrease locomotor activity in group-housed and socially isolated mice (91). In contrast to diazepam, allopregnanolone dose-dependently induces potent anxiolytic actions in socially isolated mice (16; 39).

Interestingly, the expression of $GABA_A$ receptor subunits is susceptible to changes in brain neurosteroid levels. In particular, expression of α4–containing subunits increases during progesterone withdrawal or following blockade of 5α–reductase (93). Likewise, in socially isolated mice, allopregnanolone levels decrease in several corticolimbic structures that concomitantly show changes in $GABA_A$ receptor subunit mRNA and protein expression. It would be important to determine whether social isolation directly affects the expression of $GABA_A$ receptor subunit composition or whether such changes are mediated by decreasing the levels of 5α–DHP and its binding at nuclear progesterone receptors or by allopregnanolone biosynthesis downregulation.

8. Selective brain steroidogenic stimulants (SBSSs) improve behavioral deficits in socially isolated mice

Behavioral deficits induced by social isolation in rodents include aggressive behavior (94-96). Aggression is correlated with the downregulation of corticolimbic allopregnanolone

biosynthesis (35). Upregulation of allopregnanolone levels in socially isolated mice by systemic administration or local microinfusion of allopregnanolone induces a dose-dependent amelioration of aggressive behavior of a resident mouse to a same-sex intruder (35; 77). Thus, the decrease of corticolimbic allopregnanolone levels appears to be involved in the expression of aggression.

As indicated above, SSRI antidepressants potently increase the levels of allopregnanolone in rodents and depressed humans. The effects of paroxetine and fluoxetine on allopregnanolone levels were independent from pregnenolone or progesterone levels that failed to change (34; 76). Racemic fluoxetine (R- and S-isomers) normalized the righting reflex loss induced by pentobarbital in mice by increasing corticolimbic allopregnanolone levels (35-37). Of note, at the doses used, fluoxetine failed to change the behavior and allopregnanolone levels of group housed mice (35; 36). Importantly, inhibition of serotonin synthesis by treatment with p-chlorophenylalanine failed to block the behavioral effects of fluoxetine, suggesting that increasing corticolimbic allopregnanolone levels is part of the pharmacological actions of fluoxetine (76).

These observations led us to hypothesize that fluoxetine could improve the behavioral abnormalities of socially isolated mice by enhancing corticolimbic allopregnanolone biosynthesis rather than by inhibiting serotonin reuptake. This hypothesis was investigated using the R- and S-stereoisomers of fluoxetine and norfluoxetine as pharmacological tools. We expected that these drugs would stereospecifically upregulate corticolimbic allopregnanolone content but have no stereoselectivity with regard to inhibition of 5-HT reuptake. We additionally thought that doses of fluoxetine and norfluoxetine stereoisomers that increase corticolimbic allopregnanolone content might differ from those that inhibit 5-HT reuptake. Indeed (16; 35-38), fluoxetine dose-dependently and stereospecifically normalized the duration of pentobarbital-induced sedation and reduced aggressiveness, fear responses, and anxiety-like behavior at the same submicromolar doses that normalized the downregulation of brain allopregnanolone content in socially isolated mice. Interestingly, the S-stereoisomers of fluoxetine or norfluoxetine appeared to be 3 to 7 fold more potent than their respective R-stereoisomers and S-norfluoxetine was about 5-fold more potent than S-fluoxetine. Importantly, the effective concentrations (EC_{50s}) of S-fluoxetine and S-norfluoxetine that normalize the brain allopregnanolone content are 10- (S-fluoxetine) and 50-fold (S-norfluoxetine) lower than their respective EC_{50s} needed to inhibit 5-HT reuptake (35-38). Remarkably, the SSRI activity of S or R-fluoxetine and of S or R-norfluoxetine was devoid of stereospecificity (35; 36). Hence, this study demonstrated that neither the behavioral action nor the normalization of corticolimbic allopregnanolone content by S-fluoxetine and S-norfluoxetine is related to their intrinsic SSRI activity.

9. A novel promising therapy for anxiety disorders and PTSD

In the pathophysiology of depression and PTSD, a GABAergic neurotransmission dysfunction could at least in part be involved in the symptomatology of these disorders. Decreased GABA levels and reductions in $GABA_A$ and $GABA_B$ receptor binding and/or sensitivity have been found in depressed patients (97; 98). In PTSD, decreased frontal lobe benzodiazepine receptor binding (99; 100) and decreased plasma GABA levels (101) have been demonstrated. These changes were most consistently and profoundly observed among treatment resistant patients. Benzodiazepines have not been found to effectively treat PTSD (10-12) and SSRIs sertraline and paroxetine are the only medications currently approved by

the Federal Drug Administration (FDA) for the treatment of PTSD. However, their effect sizes are modest (102-105), or even ineffective (106). In patients who cannot adequately synthesize allopregnanolone and in whom administration of an SSRI (or SBSS) is ineffective, the administration of an allopregnanolone analog (e.g. 107, 108), such as ganaxolone may offer a therapeutic alternative. A multisite Phase II trial of the efficacy and safety of ganaxolone in PTSD is currently been tested. Other medications that increase plasma allopregnanolone levels by a different mechanisms than the SSRIs also may be effective in PTSD (109-111).

The findings that the socially isolated mouse expresses decreased levels of allopregnanolone, as well as changes in the expression of several $GABA_A$ receptor subunits in corticolimbic structures that regulate cognition, anxiety, PTSD, and depression suggests that the *socially isolated mouse model* may be useful in investigating new molecules designed to improve behavioral deficits characterized by $GABA_A$ receptor signal transduction dysfunction (reviewed in 16; 38; 73).

Hence, as in PTSD patients, the socially isolated mouse fails to respond to sedative and anxiolytic benzodiazepines. Our studies demonstrate that allopregnanolone or S-norfluoxetine -at nonserotonergic doses- infused into the basolateral amygdala potently increase allopregnanolone biosynthesis in target corticolimbic areas including the hippocampus, basolateral amygdala, and frontal cortex (77) and exert a strong anti-anxiety, anti-fear, and anti-aggression effect (35-38; 72; 77).

Neurosteroids lack $GABA_A$ receptor subunit selectivity and the functional $GABA_A$ receptor binding characteristics of benzodiazepines. Thus, this suggests that allopregnanolone, its analogs, or molecules that stimulate allopregnanolone biosynthesis might be advantageous over benzodiazepines in a scenario of neurosteroid downregulation and changes in $GABA_A$ receptor subunit subtypes. Despite benzodiazepines, allopregnanolone activates $GABA_A$ receptors incorporating α4, α6, and δ subunits in combination with γ and β subunits (64-66). Thus, allopregnanolone or SBSSs improve anxiety, fear, and aggressiveness when benzodiazepines fail. Of note and in contrast to benzodiazepines, both allopregnanolone and SBSS molecules decrease anxiety, fear, and aggression at concentrations that fail to be sedative (16; 35; 39; 77).

New SBSS molecules that fail to exert any significant SSRI activity but increase corticolimbic allopregnanolone levels and thereby improve behavioral symptoms in mouse models of anxiety and depression. The high potency and stereospecificity of these drugs in reducing behavioral deficits and in normalizing brain allopregnanolone content suggest that they may affect specific targets for regulating neurosteroidogenesis. The finding that protracted social isolation affects the expression of 5α-reductase in corticolimbic structures, but fails to change the expression of 3α-HSD, as well as the finding that brain progesterone levels don't change in socially isolated mice suggest that a mechanism involving 5α-reductase is responsible for the decrease of corticolimbic allopregnanolone content. This is further supported by the fact that 5α-reductase is the rate-limiting step-enzyme in allopregnanolone biosynthesis from progesterone (73). Hence, these data suggest that fluoxetine and norfluoxetine mediate upregulation of corticolimbic allopregnanolone levels by a direct action on 5α-reductase. However, *in vitro* studies by Griffin and Mellon (112) showed that fluoxetine, paroxetine, and sertraline failed to activate 5α-reductase and instead, directly activated 3α-HSD by decreasing its K_m for 5α-DHP, thereby facilitating an accumulation of allopregnanolone (112). The hypothesis that neurosteroidogenic antidepressants activate 3α-

HSD is also suggested by the finding that fluoxetine accelerates the rate of allopregnanolone accumulation during incubation of brain slices with 5α-DHP (34). Furthermore, progesterone levels in group-housed and socially isolated mice are not affected by fluoxetine administration, suggesting that the SSRI/SBSSs impact neurosteroidogenesis downstream from progesterone (34; 76). On the other hand, experiments by Trauger and collaborators (113) were inconsistent with the hypothesis that fluoxetine and paroxetine directly activate 3α-HSD. The finding that low doses of the S isomers of fluoxetine or norfluoxetine increase corticolimbic levels of allopregnanolone in socially isolated mice, but fail to change levels in group-housed mice, suggests that 5α-reductase and/or 3α-HSD may become more susceptible to the effects of SBSSs during isolation (reviewed in 38). Investigations at the molecular enzymatic level will clarify whether social isolation and neurosteroidogenic agents change the kinetics of 5α-reductase and/or 3α-HSD.

Other feasible pharmacological targets to enhance neurosteroidogenesis include the translocase protein (18 kDa) or TSPO, previously called mitochondrial peripheral benzodiazepine receptor or PBR (114). TSPO represents the starting point and an important rate-limiting step in neurosteroidogenesis. It gives access to neurosteroids in the brain by regulating the entry of cholesterol into the inner mitochondrial membranes and its conversion to pregnenolone by P450scc, which is located in the inner mitochondrial membrane (29; 114). A cascade of enzymatic processes then take place in the cytosol, resulting in the production of neuroactive steroids, including pregnenolone sulfate, DHEAS (though apparently not in human brain (115)], THDOC, and allopregnanolone (reviewed in 31).

New molecules that bind with high affinity to TSPO have been recently investigated. These drugs are able to exert important anxiolytic effects but are devoid of the unwanted side effects associated with benzodiazepines, including over-sedation and tolerance (28; 29). In mouse models, TSPO agents have been shown to potently increase pregnenolone levels in the hippocampus and cortex, as well as to induce anxiolytic effects (116-119). TSPO ligands include XBD173 and etifoxine, which have proven to be highly efficacious anxiolytic and antidepressant drugs in a number of behavioral tests (29; 30). The anxiolytic and antidepressant effects of these agents were related their ability to increase neurosteroid biosynthesis, as confirmed by studies in which key enzyme blockers for neurosteroid biosynthesis, including finansteride and trilostane (56; 30), were used. TSPO ligands have recently showed promising therapeutic effects in clinical studies (29; 30).

10. Closing remarks

The new class of drugs, the SBSSs (selective brain steroidogenic stimulants) -whose mechanism of action involves the stimulation of neurosteroidogenesis with the goal of increasing brain allopregnanolone levels- has emerged as a new therapeutic strategy for the treatment of psychiatric disorders associated with a downregulation of brain allopregnanolone biosynthesis. These disorders include anxiety, depression, and PTSD.

In comparison to benzodiazepines, the SBSSs are more efficacious as well as devoid of the unwanted side-effects induced by benzodiazepines. Allopregnanolone pharmacology involves the allosteric modulation of GABA action at $GABA_A$ receptors, which is broader than that of benzodiazepines, which fail to modulate $GABA_A$ receptors containing α4 and α6 subunits. Hence, selective stimulation of allopregnanolone biosynthesis may avoid the therapeutic hindrances caused by the formation of benzodiazepine-resistant $GABA_A$

receptors with altered subunit composition, such as may occur in stress-related psychiatric disorders (reviewed in 120). Thus, novel SBSS drugs that specifically increase corticolimbic allopregnanolone biosynthesis appear to be a novel promising pharmacological class of future drugs for the treatment of anxiety disorders, depression, and PTSD.

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